SYMBOLAE BOTANICAE UPSALIENSES XIX: 3
ARBETEN FRÅN BOTANISKA INSTITUTIONERNA I UPPSALA
UTGIVNA AV PROF. E. MELIN OCH PROF. J. A. NANNFELDT

STUDIES IN THE PRODUCTION OF AUXINS AND OTHER GROWTH STIMULATING SUBSTANCES BY EXOBASIDIUM

BY

SVEN-OLOV NORBERG

UPPSALA 1968
A.-B. LUNDEQUISTSKA BOKHANDELN
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SYMBOLAE BOTANICAE UPSALIENSES

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SYMBOLAE BOTANICAE UPSALIENSES

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PRINTED MARCH 21, 1968, BY

ALMQVIST & WIKSELLS BOKTRYCKERI AB

UPPSALA, SWEDEN

CONTENTS

Acknowledgements 5

I. Introduction 7

II. Materials 8

III. Methods 11

A. Extraction methods 11
a. To obtain auxins 11
b. To obtain cytokinins 12
c. To obtain gibberellins 12

B. Description of the chromatography methods and the reagents 13

C. Spectrophotometric measurements 14

D. Bioassay procedures 15
a. *Raphanus* test for auxins and cytokinins 15
b. *Phaseolus* test for auxins, cytokinins and gibberellins 16
c. Wheat coleoptile test for auxins 16
d. Wheat leaf test for gibberellins and auxins 17
e. Test for gibberellins with dwarf mutants of *Zea mays* 17
f. Callus test for cytokinins 17
g. Oat leaf test for cytokinins 18
h. Wheat root test for cytokinins 19

IV. Investigations with *Raphanus* leaves as test object 20

A. Studies of the effects of known growth substances on leaves 20

B. Leaf growth experiments with filtrate from fungal cultures 24

C. The effects of chromatographed ether extracts on *Raphanus* leaves . . 28
a. Extracts of NH_4NO_3 media 28
b. Extracts of tryptophan media 30
c. Extracts of tryptamine media 34

V. Studies of the auxin metabolism in *Exobasidium* 35

A. Auxin metabolism on NH_4NO_3 medium 35
a. Introduction 35
b. Wheat coleoptile tests 36
c. Wheat leaf tests 39
d. *Phaseolus* tests 40
e. Chromatography data 40
f. Synthesis of tryptophan 43

B. Auxin metabolism on N–Z case, casamino acids, yeast extract and maize leaf extract 45
C. Auxin metabolism on tryptophan medium 48
a. Results of long term experiments 48
b. Results of short term experiments 62
D. Auxin metabolism on indolepyruvic acid medium 68
E. Auxin metabolism on indoleacetaldehyde medium 69
F. Auxin metabolism on tryptamine medium 71
G. Auxin metabolism on indolelactic acid medium 77
H. Auxin metabolism on tryptophol medium 77
I. Auxin metabolism on indolepropionic acid and indolebutyric acid media 78
J. Auxin metabolism on indoleacetonitrile medium 79
K. Auxin metabolism on indoleacetamide medium 80
L. Auxin metabolism on indoleacetic acid medium (breakdown of IAA) 81
M. Auxin metabolism on indolealdehyde medium 84
N. Auxin metabolism on IAA ethyl ester medium 86
O. Discussion of the auxin metabolism in *Exobasidium* 86
P. The auxin content of extracts from plant material infected by *Exobasidium* 90
VI. Investigations of the ability of *Exobasidium* to form phenyllactic acid 95
VII. Investigations of the ability of *Exobasidium* to form gibberellins . . . 96
VIII. Investigations of the ability of *Exobasidium* to form cytokinins . . . 99
A. Results of callus tests 99
B. Results of oat leaf tests, wheat root tests and spectrophotometric measurements 100
Summary 107
References 109
Formulae and abbreviations of substances named 114
Colour reactions with some of the reagents, and Rf values in different solvents 116
Plates 1–4 119

ACKNOWLEDGEMENTS

This investigation has been carried out at the Institute of Physiological Botany, University of Uppsala and I am very grateful to its head, Professor Nils Fries for his support, help and interest in my work.

To all my friends and colleagues at the Institute I wish to express my thanks for criticism and help. Especially, I am greatly indebted to Docent K.-R. Sundström for providing me with pure cultures of *Exobasidium* and for many valuable discussions.

It is a pleasure to acknowledge the skilful technical assistance of Miss Agneta Ottosson and Mr Åke Danielsson.

Many thanks are due to Dr. Margaret Jarvis for help with the translation of my manuscript and to Mrs Baiba Byberg for help with the preparation of the diagrams.

I also wish to express my warmest gratitude to my wife, Anna-Stina Norberg, for her continuous encouragement and support during the work.

Finally, I hereby gratefully acknowledge financial support from the University of Uppsala and "A. G. och Ingeborg Eliassons fond".

Uppsala, July 1967.

Sven-Olov Norberg

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I. INTRODUCTION

The genus *Exobasidium* belongs to the *Basidiomycetes,* but its taxonomic position within this group of fungi is uncertain. Since 1867, when Woronin described the first species, many new ones have been discovered. It is characteristic of them all that they live parasitically on leaves, stems or flowers of woody dicotyledonous plants. They mostly attack species of the family *Ericaceae,* but are also found on plants of other families. The mycelium of *Exobasidium* grows intercellularly in the infected parts of the plants and is the cause of more or less extensive changes, mainly brought about by an increase in cell size. The extent of the attack on the host plant varies considerably according to the particular *Exobasidium* species involved. The least extensive are the hypertrophic spots (galls) caused by *E. Vaccinii* which are often seen on leaves of *Vaccinium vitis-idaea* and *Vaccinium uliginosum.* Another disease symptom is manifest in systemic infections, when the mycelium grows throughout a whole shoot. In *Cassiope tetragona,* the annual shoots are swollen and yellow. The shoots of *Andromeda polifolia* may also be infected systemically. Leaves on such shoots are purple-coloured and greatly enlarged, mainly because they are much broader. Systemic infection can also be found in *Vaccinium myrtillus, V. uliginosum* and *V. vitis-idaea.* Finally, a unique disease symptom is caused by *Exobasidium uvae ursi,* which causes witches' broom formation in its host, *Arctostaphylos uva-ursi.*

Wolf and Wolf (1952) and Hirata (1957) have previously shown that certain *Exobasidium* species are able to synthesize indoleacetic acid (IAA) on tryptophan medium. However, Göttgens (1959), in investigations with *E. azaleae,* was unable to show that this species could synthesize growth substances in the proper sense, probably to some extent because of the method used, in which there was no extraction, but the filtrate from the culture was chromatographed directly. Instead Göttgens found that the fungus synthesized mesoinositol and biotin, among other substances, and he assumed that these, together with other more specifically stimulating substances (?) played a part in the formation of the gall swellings on leaves of *Azalea indica.*

The aim of this work has been to find out which substance or substances of those produced by the various *Exobasidium* species can bring about the changes in the host plants, particularly the increased leaf growth. The auxin metabolism in *Exobasidium* has been specially studied, but other groups of plant regulators such as gibberellins and cytokinins have also been searched for.

II. MATERIALS

The *Exobasidium* cultures used in my investigations have been made available by Docent Karl-Ragnar Sundström, and are the same as those referred to in his doctorate thesis (1964). The names of the tested species and "host-races", i.e. *Exobasidium* cultures isolated from the same host species and causing similar disease symptoms, are listed in Table 1. The cultures have generally grown from one spore each and are usually of differing geographical origin, since collections have been made from south Germany up to the north Swedish mountains. The cultures have been kept in a deep-freeze on malt agar.

The cultures have been grown on a wholly synthetic medium with the following composition:

Medium 1

Solution A			Solution B		
Glucose	20 000	mg	$CaCl_2$	100	mg
NH_4NO_3	600	mg	$MgCl_2 \cdot 6\ H_2O$	410	mg
K_2HPO_4	1 800	mg	Ferricitrate	5.31	mg
Citric acid. H_2O	1 150	mg	Citric acid $\cdot\ H_2O$	5.31	mg
$Na_2SO_4 \cdot 10\ H_2O$	500	mg	$MnSO_4 \cdot 4\ H_2O$	4.43	mg
Thiamine	0.1	mg	$ZnSO_4 \cdot 7\ H_2O$	4.05	mg
Distilled water up to	1 000	ml	Distilled water	50	ml

pH = 4.9

Solutions A and B were autoclaved separately. At the time of inoculation, 0.1 ml of fungal conidia was added to 5 ml of B, and 1 ml of this mixture was then added to 20 ml of A. Erlenmeyer flasks, 100 and 300 ml, were used. The flasks were shaken continuously during cultivation so that all the cultures grew in conidial form. Cultivation temperature was 20 or 25°C, according to which temperature gave best growth for the particular culture (Sundström, 1964).

The nitrogen source, viz. NH_4NO_3, was changed or supplemented in special experiments with other nitrogen-containing compounds which might affect auxin production. These substances are mostly thermolabile and were therefor sterilized by filtration instead of by autoclaving, especially DL-tryptophan (0.750 g/l), used in many experiments.

Besides the long term experiments in sterile conditions, shorter experi-

Table 1. List of *Exobasidium* strains used in the investigation.
(According to Sundström, 1964)

The fungi were collected in the vicinity of the following places:

A = Abisko (Sweden)
St = Starnberg (Western Germany)
Sw = Schwarzwald (Western Germany)
Ö = Örnsköldsvik (Sweden)
U = Uppsala (Sweden)
H = Hok (Sweden)
V = Vansbro (Sweden)

Gr. = Pure culture recieved in 1962 from Dr. Graafland, Baarn
M = Pure culture recieved in 1962 from Dr. Montant
S = Systemic attacks on whole shoots
G = A localized leaf spot with a hypertrophic gall
SG = Shoot galls
DI = Double infection. A leaf gall on a systemically infected shoot without hypertrophy
g = A small localized leaf spot with a small hypertrophic gall
W = Witches' brooms

Species		Host	Year and place of isolation	Disease symptoms
E. angustisporum Linder	1	*Arctostaphylos alpina*	A 1959	S
	2		A 1962	S
E. cassiopes Peck	1	*Cassiope tetragona*	A 1959	S
	2		A 1962	S
E. japonicum Shir.	1	*Azalea*	1961	G
E. Karstenii Sacc. & Trott	1	*Andromeda polifolia*	A 1959	S
	2		A 1959	S
	3		Ö 1959	S
	6		St 1961	S
E. myrtilli Siegm.	1	*Andromeda polifolia*	U 1959	S
	2		St 1961	S
E. myrtilli Siegm.	2	*Vaccinium myrtillus*	A 1959	S
	4		Ö 1959	S
E. myrtilli Siegm.	2	*Vaccinium vitis-idaea*	A 1959	S
	3		Ö 1959	S
E. myrtilli Siegm.	1	*Vaccinium uliginosum*	A 1962	S
E. rhododendri Cram.	1	*Rhododendron ferrugineum*	M	
E. uvae ursi (Maire) Juel	1	*Arctostaphylos uva-ursi*	U 1959	W
	2		U 1959	W
E. vaccinii (Fuck.) Woron.	1	*Vaccinium vitis-idaea*	U 1958	G
	2		U 1959	G
	3		V 1959	SG
	6		St 1961	G
	12		H 1961	G
	17		U 1962	DI
E. vaccinii (Fuck.) Woron.	3	*Arctostaphylos uva-ursi*	U 1961	g
	4		U 1961	S
	5		U 1962	g
E. vaccinii (Fuck.) Woron.	1	*Vaccinium myrtillus*	U 1960	g
E. vaccinii (Fuck.) Woron.	1	*Vaccinium oxycoccos*	Sw 1961	g
	3		U 1962	g
E. vaccinii (Fuck.) Woron.	5	*Vaccinium uliginosum*	U 1961	g
E. vexans Mass. (B_{76})	5	*Camellia sinensis*	Gr.	

ments were also carried out, mainly to study the metabolism of various indole compounds. These experiments lasted less than 24 hours, and the nutrient medium contained no sugar source. The indole compounds (*see* p. 114) were dissolved in a few drops of 96 % ethanol, and a 10^{-3} M solution was prepared by the addition of 1/50 M KH_2PO_4 (pH 4.5). However, indoleacetaldehyde had to be dissolved in phosphate buffer at pH 7.4, to prevent it breaking down. The test flasks contained the indole compounds, which had a concentration of 2×10^{-4} M, 35 ml 1/50 M KH_2PO_4, and 2 ml fungal conidia which had previously been centrifuged and washed with the phosphate solution. Sörensen's phosphate buffer was used in pH experiments. During the incubation period the flasks stood on a rotary shaker in the dark at 25°C.

In cultivation of *Exobasidium* cultures in which the ability to produce gibberellins was to be tested, another medium (Medium 2) was used, which had been used by Stodola (1955) in similar experiments, and to which thiamine was added. This medium was used in addition to the usual nutrient solution with NH_4NO_3 and nutrient solutions in which nitrate had been replaced by organic nitrogen sources (1 g/l) such as yeast extract (Difco), casamino acids (Difco) or N-Z case (Sheffield Chemical). Its composition was as follows:

Medium 2

Distilled water	1000 ml
Glucose	20 g
$MgSO_4 \cdot 7\ H_2O$	3 g
NH_4Cl	3 g
KH_2PO_4	3 g
Thiamine	0.1 mg

In experiments to find out whether the fungi synthesize cytokinins, an asparagine medium was used, as well as Medium 1. This differed from Medium 1 in having 2.8 g/l L-asparagine instead of NH_4NO_3. Asparagine medium was used by Thimann and Sachs (1966) in their successful experiments to demonstrate cytokinins in *Corynebacterium fascians*. Their methods have also been used in the extractions and in tests to show the presence of cytokinins. The fungus was also cultivated on media containing 0.1 and 0.8 g/l adenine, alone or with NH_4NO_3, as a source of nitrogen.

III. METHODS

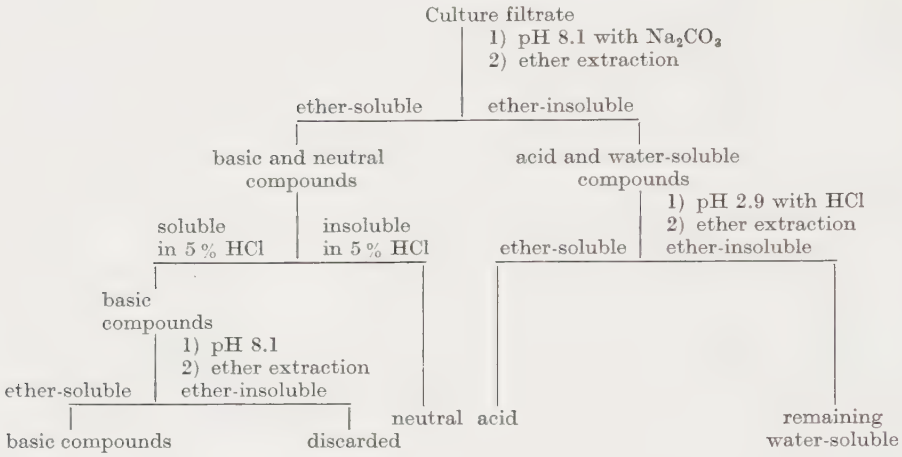
A. Extraction methods

a. *To obtain auxins*

When the fungus had grown so much that there was a dense suspension of conidia, usually after about ten days, or after a particular length of time in some experiments, the medium was in most cases extracted, if it was not to be used directly in any experiment. Extraction was mostly with peroxide- and water-free diethyl ether (produced by Uddeholm AB. Skoghall, Sweden, and containing no preservative) or in a few cases with ethyl acetate. In order that the aqueous and ether phases should separate properly after extraction, it was usually necessary to filter or centrifuge the fungal medium first, to remove the conidia. Extraction was by one of two different methods, depending on the aim of the experiment. The simplest was carried out at pH 2.9. The filtrate was shaken for two minutes with an equal volume of ether, in a separating funnel. The acid and most of the neutral fraction was retained in the ether. Before the ether was evaporated off, a pinch of dry Na_2SO_4 was added, to remove any remaining water. Evaporation was in a vacuum and continued until about 0.5 ml of ether was left. Separation of the acid and neutral fractions was by a method used by Bailey and Gentile (1962). The ether, which had been used to extract the fungal filtrate at pH 2.9, was shaken with 2% $NaHCO_3$, which removes the acid substances, leaving the neutral ones in the ether. Then the pH was adjusted to 2.9 in $NaHCO_3$ solution with HCl, and further shaking with ether transferred the acid substances to the ether phase. The acid and neutral fractions were evaporated as before. To obtain fractionation into acid, neutral and basic phases, together with ether-insoluble substances which are left in the aqueous phase, a method developed by Dannenburg and Liverman (1957) was used and the scheme on p. 12 was followed.

The samples obtained in the extractions were numbered and stored in a cold room, after a portion of the contents had been chromatographed more or less immediately. Since the extracts were retained, subsequent checks and comparisons could be made.

Extractions of plant material, particularly of leaves and shoots infected with *Exobasidium*, were also carried out. The plant material was either



macerated directly in ether or ethyl acetate or else in iced water with sand as an abrasive. After filtering, the aqueous solution was extracted with ether according to one of the above methods.

b. *To obtain cytokinins*

Kinetin in aqueous solution goes over to the ether phase, at least partially, at neutral pH (according to the Merck Index, 1960). The pH of the fungal filtrate was therefore adjusted to 7.0 and the filtrate was shaken several times with ether for five minutes. The ether fractions were put together and evaporated down to ca. 0.5 ml. The volume of filtrate extracted varied from 40–5000 ml in this and in the auxin and gibberellin extractions. Extractions have also been done with chloroform at pH 7.0 following a scheme as described by Thimann and Sachs (1966).

c. *To obtain gibberellins*

An extraction procedure described by Stowe and Yamaki (1957) was followed. The culture filtrate was treated with active charcoal. The substances adsorbed on the charcoal were eluted with basic methanol, viz. methanol made basic with NH_3. After removal of the charcoal by filtering, the methanol extract was evaporated to dryness at 50°C. The residue was taken up in 2% $NaHCO_3$, which was shaken with ether or ethyl acetate, after which the aqueous phase was acidified (pH 3.5), and any gibberellins present were taken up in ether through a new shaking procedure. The ether was evaporated down to ca. 0.5 ml, for chromatography and for use in the gibberellin test on dwarf maize mutants.

B. Description of the chromatography methods and the reagents

At first, paper chromatography was used for separating the components of the extracts. The ascending method was chosen, with a 10 hour saturation period for the atmosphere in the trough before putting in the chromatogram. Isopropanol: NH_3: water 10:1:1 was used as the solvent for the chromatography, and the path length was 30 cm.

Early on, I changed to thin layer chromatography, because of the shorter chromatography time and the ten-fold, or more, increase in detectability of the substances. The basic equipment was that developed by Stahl (1962), consisting of a spreader, glass plates 5×20 and 20×20 cm, a glass aligning tray and micropipettes. Kiselgel G, with or without UV indicator, was used as adsorbent, in layers 250 nm thick. After the kiselgel had been spread, the plates were dried in an oven at 100°C for one hour, then cooled in a desiccator over silica gel, where they were stored until they were to be used. A glass vessel 25×25 cm was used as the trough for the large plates; a 500 ml measuring cylinder which could be closed with a lid and sealed with plasticine (Terostat, type IX, Teroson-Werke, Heidelberg) for the small ones. The insides of the vessels were lined with filter paper soaked in the solvent, so that the atmosphere remained saturated during the chromatography. In most of the experiments with the small glass plates 50 ml of liquid was used, with the starting point 1 cm above the surface. The solvent was allowed to move 10 cm, which took less than 30 min in most of those which were used. The following solvents proved suitable in my investigations:

Chloroform: acetic acid (96 %) 95:5. S (saur) of Stahl (1962)
Chloroform: methanol: acetic acid (99–100 %) 75:20:5. Sp (saur, polar) of Stahl (1962)
Methyl acetate: isopropanol: NH_3 (25 %) 45:35:20. A (alkalisch) of Stahl (1962)
Isopropanol: NH_3 (25 %): water 85:5:10.
Petroleum ether: chloroform: ethanol (96 %) 50:30:20.
Isopropylether: acetic acid (96 %) 95:5. 1 of MacMillan and Suter (1963)
Chloroform: CCl_4: methanol 50:40:10. I of Ballin (1964)
Ethyl acetate: isopropanol: water 65:24:11. III of Ballin (1964)
Chloroform: CCl_4: methanol 50:25:25. IV of Ballin (1964)
Chloroform: ethanol (96 %) 65:35. V of Ballin (1964)
Ethanol (50 %), HAc-saturated atmosphere.

Immediately after chromatography, and after the plate had been illuminated with an UV lamp (at 2537 and 3650 A) and the fluorescent spots had been marked, the chromatogram was sprayed, except where the spots had been scraped off first for use in bioassays. The following reagents were used to show the various indole compounds:

REAGENTS:

Ehrlich's: consisting of 1 g 4-dimethylaminobenzaldehyde dissolved in 50 ml conc. HCl, to which 50 ml 96 % ethanol was then added. This was the reagent most often used, and unless otherwise stated the colours noted were obtained by spraying with it.

Dimethylaminocinnemaldehyde: prepared in the same way as the above.

Prochaska's: a freshly prepared mixture of 10 ml formaldehyde (35 %), 10 ml HCl (25 %) and 20 ml ethanol (96 %). After heating for 5 min at 100°C, fluorescent spots were obtained on illumination with UV light at 3650 A. These were intensified by passing nitromuriatic acid vapour over the chromatogram.

2,4-dinitrophenylhydrazine: a saturated solution in 2 N HCl, to which was added an equal volume of ethanol (96 %) immediately before use.

van Eck's: 1 g benzidine (basic) dissolved in 100 ml acetic acid (96 %). Gives a yellow colour with indole aldehyde.

Salkowski: by Gordon–Weber's method, described by Linser and Kiermayer (1957), together with several other reagents and extraction methods. The reagent consists of 1 ml 0.5 M $FeCl_3$ and 50 ml 35 % $HClO_4$. 2 ml reagent was added to 1 ml aqueous solution of the sample to be tested.

Some chromatograms were photographed after the spots had developed fully—the slowest took 20 h. Otherwise they were traced, or stored (after 10.9.64). After that date I began to use a method which makes it possible to store chromatograms. A suspension of a polymer dispersion with the trade name "New" Neatan (Merck) was sprayed on to the chromatogram and allowed to dry, and then transparent foil was stuck on. When the plate was then dipped in water the foil + the kiselgel layer came away from the glass. When this was dry it could be stuck on paper and kept. The colours were retained and sometimes became more intense rather than fading.

C. Spectrophotometric measurements

To obtain the UV absorption spectra for various substances certain zones of the chromatograms have been scraped off and eluted with methanol or, exceptionally, with ether. After centrifuging, measurements were made between 225 and 310 nm with a Zeiss Spectrophotometer. Readings were usually at 5 nm intervals, but were closer together around especially interesting points, e.g. at 280–290 nm, where most indole compounds have absorption maxima and points of inflection. The absorption has been expressed as the *Z* value, which is the extinction value × 1000.

IAA concentrations have been determined spectrophotometrically after the addition of a solution of Salkowski reagent. The intensity of the resulting red colour was measured at 525–530 nm after about 30 min.

D. Bioassay procedures

a. *Raphanus test for auxins and cytokinins*

I had already used leaves of *Raphanus sativus* (radish) in earlier growth experiments. These experiments were under sterile conditions and could go on for several days. Because of the disadvantages of the method which became apparent, including the difficulty of obtaining sufficiently homogeneous leaves, I have now used a method more or less the same as that developed by Kuraishi and Okumura (1956). The plant material was grown in unsterile conditions and put in the dark 24 hours before use, to decrease the leaf auxin content. Leaf discs with a diameter of 5 mm were then punched out from the specially selected first real leaves, with a cork borer. These were floated in distilled water for one hour, dried between filter papers, weighed, and transferred to the dishes with test solutions, where they floated on the surface. Experiments have been done using 15 leaves or 40–50 leaves per dish, depending on the availability of leaves, with 2 to 5 replicates. The dishes stood in a thermostat at 25°C with illumination of ca. 1000 lux. The light source consisted of five Philips fluorescent tubes; three TL 40 W/55, one TL A 40 W/05 and one TL 40 W/32. After 24 hours the leaves were blotted again, weighed to the nearest 0.1 mg and dried in an oven at 100°C, unless they were to be transferred to new test solutions with the same composition as before. By this means the duration of the experiment could be increased to several days, without any significant infection being observed in the dishes.

The following leaf nutrient solution was used in the experiments. In choosing the source of sugar for the leaves, account was taken of Yarwood's (1946) opinion that saccharose is preferable to glucose for cultivating detached leaves, because undesirable microorganisms grow slower.

Medium 3

$Ca(NO_3)_2 \cdot 4\ H_2O$	0.25 g
KCl	0.25 g
$MgSO_4 \cdot 7\ H_2O$	0.25 g
KH_2PO_4	1.4 g
$Na_2HPO_4 \cdot 2\ H_2O$	1.8 g
$Na_2SO_4 \cdot 10\ H_2O$	0.25 g
NH_4NO_3	0.10 g
Saccharose (omitted in some experiments)	20 g
Distilled water up to	1000 ml

In experiments with fungal filtrate, 16 ml leaf nutrient solution and 0–4 ml fungal filtrate were placed in the test dishes. The volume was always made up to 20 ml with fungal medium in which no fungus had grown.

b. *Phaseolus test for auxins, cytokinins and gibberellins*

This is also a leaf test, developed by Wheeler (1960), in which are used selected plants of the dwarf variety "Canadian Wonder" of *Phaseolus vulgaris*. Seeds of uniform size were planted in damp sand in closed containers and left for 6 days at 25°C in the dark. Discs, 5.6 mm diameter, were then punched out of the first real leaves, in weak green light. Nine leaf discs were laid on 3 cm × 3 cm pieces of filter paper in 5 cm diameter petri dishes containing 0.6 ml leaf nutrient solution in which was the substance to be tested (usually scraped from a chromatogram). The nutrient solution had the following composition:

Medium 4

K_2HPO_4	1.794	g
Citric acid · H_2O	1.019	g
Saccharose	10	g
Distilled water up to	1000	ml

pH = 5.0

Three replicates of each test solution were used. Duration of the experiments was 24 hours. The dishes stood in the dark at 25°C. The final diameter of the leaves was measured at 10× magnification at right angles to the main vein with an accuracy of 0.1 mm.

c. *Wheat coleoptile test for auxins*

This straight growth test has been developed by Nitsch and Nitsch (1956), who proceeded as follows. Wheat grains were soaked in tap water for two hours, then arranged in parallel rows on moist filter paper in 20 cm petri dishes with all the embryos orientated similarly. The dishes were placed in a drawer in such a way that they were at about 45° to the horizontal, with the embryos pointing downwards. By this means straight coleoptiles were obtained. They were cultivated in the dark at 25°C for just under three days and were then an average of 2.5 cm long. Coleoptiles of the same length were selected and a piece 5 mm long was cut off, 3 mm down from the tip, using a cutting apparatus designed for the purpose. The coleoptiles, including the first leaves, were floated on distilled water for an hour before blotting between filter papers and tranfer to the test dishes, ten coleoptiles to each dish.

All work with the coleoptiles was in weak green light. The standard solution used for the coleoptiles was the same as for the *Phaseolus* leaves (Medium 4). After incubation for 20 hours in the dark on a slow shaker, which gave better growth, the length of the coleoptiles was measured to the nearest 0.1 mm. For each dish the two highest and the two lowest readings were discarded before calculation of mean values. This was done to eliminate distortion of the final value caused by single damaged coleoptiles. A similar procedure was used in the *Phaseolus* and wheat leaf tests (*see* below).

d. *Wheat leaf test for gibberellins and auxins*

The plant material for this bioassay, which was described by Radley (1958), was the same as for the coleoptile test above, but the coleoptiles were 4 to 5 cm long. The coleoptile, with the included leaf, was cut off close to the grain, then the same "guillotine" was used as in the coleoptile test, but the basal part of the wheat leaf was used, between 4 and 8 mm from the base. This includes the greater part of the meristematic region of the leaf. Other procedure was exactly the same as described for the coleoptile test.

e. *Test for gibberellins with dwarf mutants of Zea mays*

This bioassay, carried out according to Phinney and West's directions (1961), was used as a gibberellin test. Seeds of *Zea mays* dwarf mutant 5 (given to me by Fil. lic. Lennart Boo) were planted in boxes of wet sand after soaking for 20 hours. The boxes were put in a thermostat at 30°C and watered well so that the seeds would germinate similarly. Only 25% of the seedlings were dwarf mutants and all normal seedlings were cut off before the experiment began, otherwise they caused crowding in the boxes. After seven days the dwarf mutants were of a suitable size for experiments. At that stage the first leaf began to unfold from the coleoptile, forming a bowl-shaped part which could hold 0.1–0.2 ml of test solution. Test solutions were known concentrations of gibberellic acid or scrapings from chromatograms added to 1 ml of a 0.1% solution of Tween 20 in water. 0.1 ml of test solution were added into the unfolding leaves on alternate days until the end of the experiment after about 10 days. During this period the boxes stood in a thermostat with illumination at 30°C. The plants were then pulled up and the lengths of the first, and first+second, leaf sheathes were measured in mm.

f. *Callus test for cytokinins*

Seeds of *Nicotiana tabacum* var. "Alida" were surface-sterilized with alcohol, mercuric chloride and calcium hypochlorite (the most commonly used

method in the Institute). The seeds were placed on agar medium, where only a few germinated, but from these it was possible to isolate a culture of stem callus, with the help of Fil. dr. Tage Eriksson. After re-inoculating several times, the test flasks were inoculated with the callus. The size of the inoculum was 5 to 10 mg. Erlenmeyer flasks, 100 ml, were used, each containing 40 ml of Medium 5 which is the "revised medium 1964" as worked out by Linsmaier and Skoog (1965). Except in certain controls, the kinetin was omitted and the extract to be tested was substituted.

Medium 5

NH_4NO_3	1650 mg/l	$Na_2MoO_4 \cdot 2\ H_2O$	0.25 mg/l
KNO_3	1900 mg/l	$CuSO_4 \cdot 5\ H_2O$	0.025 mg/l
$CaCl_2 \cdot 2\ H_2O$	440 mg/l	$CoCl_2 \cdot 6\ H_2O$	0.025 mg/l
$MgSO_4 \cdot 7\ H_2O$	370 mg/l	IAA	2 mg/l
KH_2PO_4	170 mg/l	Kinetin	0.03 mg/l
Na_2EDTA	37.3 mg/l	Thiamine	0.4 mg/l
$FeSO_4 \cdot 7\ H_2O$	27.8 mg/l	myo-Inositol	100 mg/l
H_3BO_3	6.2 mg/l	Saccharose	30 g/l
$MnSO_4 \cdot 4\ H_2O$	22.3 mg/l	Agar	10 g/l
$ZnSO_4 \cdot 4\ H_2O$	8.6 mg/l		
KI	0.83 mg/l	pH adjusted to 5.6	

During the period of the experiment the flasks stood in the dark at 25°C, or in a thermostat at 27°C with continuous illumination of ca. 1000 lux from the same light source as described above.

g. *Oat leaf test for cytokinins*

In addition to experiments with callus tissue, two other methods were employed, subsequently, to show the presence of cytokinins in the fungal extract. One of these was the oat leaf test, done in the way described by Gunning and Barkley (1963). The uppermost 6–8 cm of the first leaf of 6–10 days old oat seedlings were used in the test. The leaves were arranged in groups of three on microscope slides in 9 cm petri dishes, which contained 3 ml water. The test solutions, which contained 0.2% Tween 80, were applied in small drops (20 μl) to the middle of the adaxial sides of the leaves. The dishes were then stood in the dark at 25°C. After 3–4 days the leaves were completely yellow, except for the areas under the drops containing kinetin, where chlorophyll was still present. At high kinetin concentrations there was a broad green zone around the drops. Test solutions included known substances, extracts from fungal cultures and eluted chromatogram zones.

The oat leaf test was done quantitatively by using a 15 mm diameter cork

borer to cut the zone round the treated area. The leaf discs were then crushed and extracted with 5 ml acetone. The amount of chlorophyll extracted was measured by determining extinction at 660 nm.

Other leaves were tried as experimental material in this drop test. Wheat leaves were sometimes even more sensitive than oat leaves, but were more difficult to work with, whereas leaves of *Zea mays* and *Phaseolus vulgaris* did not react noticably.

h. *Wheat root test for cytokinins*

Because of their high sensitivity to kinetin, wheat roots were used in some experiments. Like roots of *Allium cepa* (McManus, 1960) and *Pisum sativum* (Manos, 1961), wheat roots react to kinetin by the formation of various abnormalities, including the typical so-called "c-swellings", which are thickenings just behind the root apex. In addition, the roots curve and become claw-like in appearance (*see* Photo 2). Two-day-old wheat seedlings, with roots ca. 2 cm long, were fixed in the holes in the perforated lids of dishes containing the test substance together with 66 ml Sörensen's phosphate buffer (0.001 M, pH 5.9), $MgSO_4$ (0.0005 M), $Ca(NO_3)_2$ (0.001 M), KNO_3 (0.001 M) and Fe–EDTA (0.00001 M). There were five or six seedlings in each dish. The dishes were illuminated continuously at 25°C. After 24 hours the roots were examined and photographed.

IV. INVESTIGATIONS WITH *RAPHANUS* LEAVES AS TEST OBJECT

A. Studies of the effects of known growth substances on leaves

The first experiments were with radish leaves, beginning with preliminary test of the growth of the leaves in various environments and their reaction to the addition of known concentrations of growth substances. Growth was expressed as a percentage of the weight at the beginning of the test. The values are means for all the replicates. Experiments in which the values for the replicates were more than 2% different from the mean were discarded.

A normal growth curve is shown in Fig. 1. The leaves were floated on a leaf nutrient solution containing 2% saccharose and were illuminated. After a lag phase, the fresh weight of the leaves increased fairly linearly.

To find out the effect of the sugar on leaf growth, and, in particular, the extent to which small differences in concentration might affect the result, an experiment was carried out in which leaves were floated on solutions containing various concentrations of glucose for three days (Table 2). As expected, the result showed that growth was increased by the addition of sugar, but also that variations in the concentration of sugar over 0.5% had no clear effect on the fresh weight of the leaves, whereas the dry weight was markedly affected. This means that the small differences in sugar concentration, which might arise in experiments in which there were additions from fungal cultures could not be of any significance. In these experiments, filtrate, in which the glucose concentration might be somewhat variable, because some of the glucose had been taken up by the fungus, was added to a larger volume of leaf nutrient solution containing 2% saccharose.

The following results have been obtained from experiments to find out whether the leaves respond to known growth-stimulating substances, and, if so, to measure the response. Table 3 shows the results of an experiment in which leaf discs floated on solutions in dishes for three days, except when they were being weighed. The leaf nutrient solution contained various concentrations of IAA, but no sugar. The solutions were not changed during the experiment. After 24 hours, there had been a slight stimulation of growth

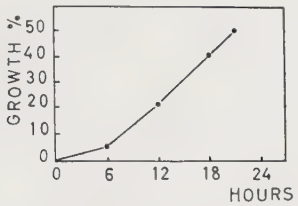


Fig. 1. The growth (fresh weight) of *Raphanus* leaf discs in standard conditions.

of the leaf discs in the dishes with the highest concentrations of IAA. The maximum stimulation was at 10^{-6} M IAA. After 48 hours the maximum stimulation had shifted to 10^{-4} M; and after 72 hours the effect had disappeared. This was because IAA rapidly breaks down, as was shown by tests with Salkowski reagent. After 48 hours there was no red colour at all produced with this reagent, showing that in dishes with an original IAA concentration of 10^{-4} M the concentration had decreased at the least to less than 10^{-5} M.

Table 2. The effect of different concentrations of glucose on *Raphanus* leaf discs. The leaf nutrient solution was changed daily.

Glucose %	% growth (fresh weight) after			Dry weight, 72 h, mg
	24 h	48 h	72 h	
0	31.2	75.5	126.4	29.6
0.5	53.0	117.1	181.0	63.6
1	51.9	115.8	177.4	78.3
2	52.3	120.1	184.0	103.8

Table 3. The effect of different IAA concentrations on *Raphanus* leaf discs. The sugar was omitted from the leaf nutrient solution, which was not changed.

Conc. IAA. M	% growth (fresh weight) after		
	24 h	48 h	72 h
0	14.9	40.9	64.2
10^{-8}	14.3	38.7	63.3
10^{-6}	18.2	41.7	66.9
10^{-4}	17.6	43.5	65.6
Salkowski test, 10^{-4}	red	0	0

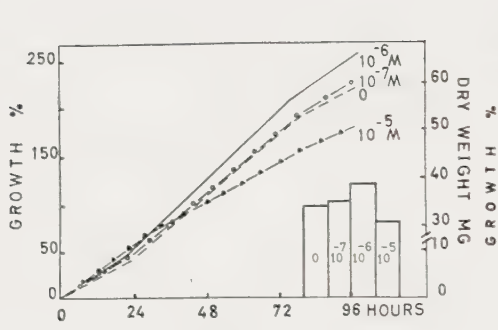


Fig. 2.

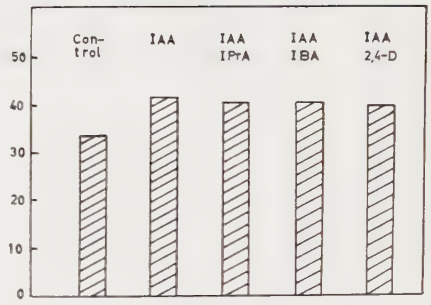


Fig. 3.

Fig. 2. The effect of IAA on growth of *Raphanus* leaf discs. Leaf nutrient solution was changed daily. Fresh weights are shown in curves; dry weights after 96 hours in columns.

Fig. 3. The response of *Raphanus* leaf discs to optimum concentrations of known auxins after 30 hours. No sugar in the leaf nutrient solution.

In order to study the effect of IAA on the leaves over a long period, it was necessary to change the IAA solutions every day (Fig. 2). After 24 hours, it was apparent that leaves on IAA concentrations of 10^{-6} and 10^{-5} M had been stimulated most in comparison with the controls. After a longer time it became clearer that the maximum stimulation of the leaves was in response to 10^{-6} M IAA; that 10^{-5} M IAA was too high a concentration and caused inhibition of growth; and that 10^{-7} M was too low a concentration and gave about the same results as the control. The dry weights were measured after 96 hours. They showed that the increases in fresh weight were not wholly attributable to increased water uptake and a consequent increase in cell size, since there was a dry weight increase at the optimum IAA concentration.

Table 4. The effect of known growth substances on *Raphanus* leaf discs. No sugar in the leaf nutrient solution.

	% growth for the following concs.				
Substance	0	10^{-8}M	10^{-6}M	10^{-4}M	Hours
2,4-D	108.1	117.1	124.8	82.8	73
NAA	54.5	57.9	61.2	43.0	47
IPrA	58.7	58.8	65.8	56.8	46
IPrA	160.0	161.9	181.9	146.7	97
IBA	57.3	58.0	61.5	59.1	49

Table 5. The effect of kinetin on the growth of *Raphanus* leaf discs. No sugar in the leaf nutrient solution.

Conc. kinetin mg/l	% growth (fresh weight) after	
	30 h	66 h
0	47.1	125.1
0.01	53.7	134.3
0.1	60.3	149.6
1.0	68.1	156.1
10.0	72.2	164.0
50.0	65.1	150.9

Several later experiments confirmed more and more clearly that IAA concentrations as high as 10^{-5} M are required to give a maximum effect after so short an experimental period as 24 hours.

The sensitivity of the leaves to IAA was also tested both in the light and in the dark. The results showed clearly that the biggest growth response was in the light. Subsequent tests using *Raphanus* leaves were therefore done in the light.

The effect of other auxins on leaf growth has also been investigated. Results in Table 4, for the synthetic substances 2,4-D (dichlorophenoxyacetic acid) and NAA (naphthylacetic acid), show clearly that these have the same effect as the natural auxin, IAA. The increase in fresh weight was greatest at 10^{-6} M, and higher concentrations caused inhibition. The effect could be studied over a long period without changing the solutions in the dishes, because 2,4-D and NAA are not as photo-labile nor as sensitive to oxidase activity as IAA.

Table 4 also shows that indolepropionic acid and indolebutyric acid, which are weaker but more stable auxins than IAA, cause effects in the leaves, at about the same concentrations. The formulae of these compounds, together with others used in the work, are listed on pp. 114–115.

The same growth-stimulation of leaves in the dishes was obtained in tests of IAA alone, or in combination with some of the other auxins in optimum concentrations (Fig. 3). There was no detectable additive effect of the different substances and all the results were very similar.

Similar growth experiments were done with kinetin, which is one of a completely different group of growth-stimulating substances. The results (Table 5) are in complete agreement with those of Kuraishi and Okumura's (1956). The growth of the leaf discs was stimulated by kinetin. There was

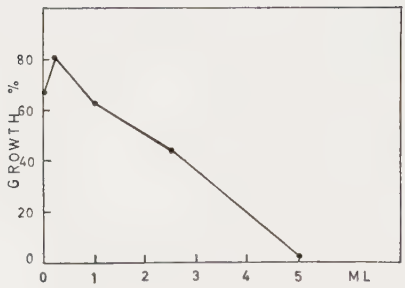


Fig. 4. The effect on the fresh weight of intact sterile leaves of *Raphanus* after 30 hours of 0–5 ml (of total 10 ml) culture filtrate from *E. Karst.* 3 grown on NH_4NO_3 medium (11 days).

a detectable effect even of so low a concentration as 0.1 mg/l; the maximum effect was at 10 mg/l and the effect decreased at higher concentrations.

Both the auxin and the kinetin experiments demonstrated that the test method using *Raphanus* leaves could be employed to detect any growth-stimulating substances in fungal media. However, it is very important that only young leaves are used. No fresh weight increase could be detected in older leaves in which cell division ceased. This agreed with Dale's (1966) investigations.

B. Leaf growth experiments with filtrate from fungal cultures

Raphanus leaves were used as test material in these experiments. The filtrate was usually from cultures of *E. Karstenii* 3, which is a systemic species infecting *Andromeda polifolia* and causing enlargement of the host leaves.

Table 6. The effect of 6 to 12 days old cultures of *E. Karst.* 3 on *Raphanus* leaf discs, when NH_4NO_3 was the nitrogen source in the fungal medium. A final volume of 20 ml in the test dishes.

Age of the culture in days	% growth (fresh weight) and dry weight (mg) with			
	0 ml filtrate	1 ml filtrate	2 ml filtrate	4 ml filtrate
6	47.5 (23.3)	53.2 (24.8)		56.4 (25.4)
8	49.7 (28.5)		57.3 (28.8)	
9	56.3 (29.5)	60.2 (30.9)		68.6 (32.1)
10	51.1 (23.9)		59.7 (25.5)	
11	46.7 (26.2)	55.7 (27.4)		58.4 (27.0)
12	65.0 (29.6)	73.7 (30.5)		84.1 (31.0)

Table 7. The effect of culture filtrate from *E. Karst.* 3 on *Raphanus* leaf discs. The fungus had been cultivated on NH_4NO_3 medium for 6 days. The dishes with the discs were in a dark cold room (+4°C) for 10 hours before being placed in light at 20°C.

Volume filtrate added, ml (of total 20)	pH-value		% growth (fresh w.)	Dry weight, mg
	At the start	At the end of exp.		
0	2.9	3.0	[a]	
2	2.9	3.6	[b]	
0	4.7	5.8	68.3	33.2
2	4.7	3.9	103.4	34.1
0	6.6	6.5	57.9	35.5
2	6.6	6.0	111.7	37.4

[a] The leaves quite white.
[b] The leaves much greener.

Fig. 4 shows a representative example of the results of my earlier work (not published previously). These experiments were done with whole, sterile leaves, to which different amounts of filtrate from *E. Karst.* 3 in NH_4NO_3 medium were added. In most treatments, growth of the leaves was inhibited, particularly by the highest concentrations of filtrate. However, Fig. 4 shows that there was a slight stimulation of growth by the smallest addition (0.2 ml) of filtrate. In subsequent experiments consequently only small volumes of cell-free medium were added to the leaf nutrient solution.

Results of the subsequent experiments are summarized in Tables 6 and 9, grouped according to the age of the tested culture. Certain tendencies are apparent. Growth stimulation in treatments with small additions of culture filtrate occurred in experiment after experiment and was usually quite distinct (Table 6). Maximum values were an average of ca. 10% higher than the controls. In one or two cases, considerably greater stimulation of leaf growth was measured. Table 7, showing the results of an experiment carried out at various pH values, includes an example. At pH 6.6, growth was almost double that of the control, as could easily be seen with the naked eye. At pH 4.7, growth was somewhat less, but still very much more than the control. This very marked effect on the leaf is similar to that obtained in several previous long term (5–6 days) experiments with sterile leaves. A factor which might have affected the results of this experiment was that the leaves in the dishes stood in the cold room at +4°C for 10 hours before being moved into the illuminated, controlled-temperature cabinet. The experiment shown in

Table 8. The effect of filtrate from a 8 days old culture of *E. Karst.* 3 on NH_4NO_3 medium on *Raphanus* leaf discs. a and b placed directly in light, c and d first at +4°C for 10 hours.

Volume filtrate added, ml	pH at start	% growth (fresh w.)	Dry weight, mg
0 (a)	5.2	45.9	28.3
4 (b)	5.2	47.2	29.6
0 (c)	5.2	45.5	29.1
4 (d)	5.2	53.9	30.0

Table 8 was designed to check this possibility. The effect on growth was not as large, but it was still largest in the dishes which contained filtrate and which had stood in the cold room.

Dry weights are included in most of the tables, and they are generally highest for the leaves which showed the greatest increase in growth (fresh weight), showing that the increase was not attributable only to increased water uptake by the cells.

This growth stimulation was caused only by filtrate from fungal cultures which were not older than ca. 12 days. This figure of 12 days is of course only an approximate limit, since it is not the age of the culture *per se* which is the cause, but the growth of the fungus, which varies somewhat, according to the density of the inoculum. This is illustrated by Table 9 which shows that a dense 12-day-old culture (turbidimetrically measured at 610 nm) caused inhibition of leaf growth even when only small amounts of the medium were added, whereas a sparse 19-day-old culture of the same fungus had a definite positive effect. Filtrates from older dense fungal suspensions, at first in large amounts and later in smaller amounts, had an inhibitory effect

Table 9. The effect of 12–21 days old cultures on *Raphanus* leaf discs. *E. Karst.* 3 was grown on NH_4NO_3 medium and the densities of the cultures were measured turbidimetrically at 610 nm.

Age of the culture in days	Z-value	% growth (fresh weight) and dry weight (mg) with			
		0 ml filtrate	1 ml filtrate	2 ml filtrate	4 ml filtrate
12	1460	54.7 (19.5)	47.2 (18.1)	43.7 (17.6)	
12 (boiled)	1460	54.7 (19.5)	50.6 (18.4)	48.1 (18.2)	
15		74.8 (36.4)	74.5 (36.5)		66.8 (34.2)
19	540	69.2 (18.5)		78.8 (19.1)	
21		42.7 (21.9)	40.0 (21.0)		37.8 (20.5)

Table 10. The effect of culture filtrate on *Raphanus* leaf discs. *E. Karst.* 3 was incubated on asparagine medium for 11 days.

Volume filtrate added, ml	% growth (fresh w.)	Dry weight, mg
0	25.4	18.8
0.5	30.4	18.9
1.0	31.1	19.2
2.0	28.9	19.4

on leaf growth. This cancelled out the stimulatory effect caused by the filtrate from younger cultures. The inhibitory factor is still unknown but it is probably attributable to an accumulation of one or several toxic substances rather than too high a concentration of stimulatory substances such as auxins. Table 9 shows that the inhibitory substances are heat-labile to some extent, because the leaf growth was not depressed as much by filtrate which had been boiled. Brian *et al.* (1961), and others, have demonstrated similar inhibitory effects of filtrate from fungal cultures on leaf growth. The most important phytotoxic substance isolated by Brian *et al.* from *Fusarium equiseti* was the colourless product, diacetoxyscirpenol ($C_{17}H_{26}O_7$). One of the effects of this substance is decolorization of the leaf mesophyll, and this is also brought about by additions of large amounts of filtrate from *Exobasidium* cultures. Salemink *et al.* (1966) have recently isolated a phytotoxin produced by *Didymella applanata.* This has been shown to be a complex compound, containing d-glucose. In addition, several other fungi have been shown to produce toxins when they are grown in an artificial medium. Various types of pectolytic and cellulolytic enzymes occur, as well as the "wilt toxins" and several others included in Bollard and Matthew's review (1966).

Further experiments showed that it was not only *E. Karst.* 3 which could stimulate growth, but that *E. Karst.* 5 could also do so, as expected. Nor was it necessary for the fungus to be grown on NH_4NO_3 medium. Small additions of young cultures on asparagine also caused growth stimulation, although larger additions soon had an inhibitory effect. One ml filtrate added to 19 ml leaf nutrient solution caused the maximum effect on the fresh weight (Table 10).

Growth 13.3% higher than the control was measured as a result of the addition to the leaves of 0.3 ml filtrate from *E. Karst.* 3 which had grown on tryptophan medium for only 22 hours. A test of this filtrate with Salkowski reagent gave a weak red coloration, so that this particular stimulatory effect

Table 11. The effect of pieces of a paper chromatogram on the fresh weight of *Raphanus* leaf discs and the length of wheat coleoptiles. Ether extract (pH 2.9) of *E. Karst.* 6 on NH_4NO_3 medium for 10 days. Solvent isopropanol : NH_3 : H_2O 10 : 1 : 1.

Rf values	Salkowski reaction	Leaf discs % growth	Coleoptiles mean length, mm
Control	0	43.2	10.8
0.00–0.11	0	42.2	10.7
0.11–0.22	0	42.7	10.8
0.22–0.33	0	42.9	10.6
0.33–0.44	0	47.2	10.9
0.44–0.55	0	46.5	10.7
0.55–0.66	0	44.4	11.0
0.66–0.77	0	45.3	11.1
0.77–0.88	0	45.0	11.3
0.88–1.00	0	45.7	10.5

was probably due to IAA. The explanations of the effects when NH_4NO_3 and asparagine media were used are not so clear. Although *E. Karst.* 3 produces IAA on these media (as will be discussed later) the amounts involved are considerably less. It is therefore extremely unlikely that IAA is the only cause of the growth stimulation in this case. As shown later, other indole compounds with an auxin effect are produced, but growth substances which are not auxin-like may also be concerned.

C. The effects of chromatographed ether extracts on *Raphanus* leaves

In this type of experiment, the chromatogram was divided longitudinally into four equal parts. Each of these was subdivided and used in leaf tests, coleoptile experiments, UV measurements and tests with colour reagents.

a. *Extracts of NH_4NO_3 media*

Paper chromatography was used in the earliest experiments (for example, Table 11). The solvent was isopropanol: NH_3: water; and the reagent was Salkowski reagent, which is unfortunately not very sensitive. There were no detectable spots on the chromatogram and the sensitive coleoptile test for auxins reacted only weakly to the next-to-last zone. There was some stimula-

Table 12. The effect of chromatographed ether extract (pH 2.9) of *E. myrt.* "Andr." 2 grown on NH_4NO_3 medium for 12 days on the growth of *Raphanus* leaf discs and coleoptiles. Solvent A. Ehrlich's reagent.

Rf values	Colour with reagent	Leaf discs % growth	Coleoptiles mean length, mm
Control	0	43.0	10.0
0.00–0.11	0	42.8	9.8
0.11–0.22	0	42.4	10.1
0.22–0.33	0	45.7	10.9
0.33–0.44	blue	48.2	13.7
0.44–0.55	0	46.2	11.1
0.55–0.66	0	41.4	9.5
0.66–0.77	0	45.2	10.8
0.77–0.88	blue (weak)	48.2	11.6
0.88–1.00	0	45.2	10.0

tion of leaf growth in the dishes containing eluate from the zones Rf 0.33 to 0.44 and above.

When thin layer chromatography, and Solvent A was used, more concentrated spots were detectable and there were greater effects on the leaves (Table 12). The Rf values were about the same as in isopropanol: NH_3: water. There were two main zones on the chromatogram which caused growth stimulation, Rf 0.33 to 0.44 and 0.77 to 0.88. The greatest coleoptile growth was caused by the same zones, indicating that auxin-like substances were involved. The most consistent leaf growth stimulation was by the zone with the highest Rf value. This must be partly because supra-optimum concentrations were never reached. After chromatography in Solvent S, this active substance was in zone Rf 0.44 to 0.55 (Table 13). For this zone, leaf growth was 12% more than the control and the dry weight was also higher. In this experiment, and others, the first zone of the chromatogram also caused increased leaf fresh weight.

At this stage, it was difficult to say with certainty what the substances were which caused the growth increase of the *Raphanus* leaves. Chromatography of large volumes of extract from NH_4NO_3 media showed subsequently that in the group of fungi used in these experiments there are always two distinct spots, coloured blue-violet with Ehrlich's reagent. The lowest and most distinct of the spots was immediately suspected to be IAA, and this was later confirmed. The identity of the uppermost blue spot was shown only much later.

Raphanus leaves have also been used to test the same spots scraped from

Table 13. The effect of zones of a chromatogram of ether extract (pH 2.9) of *E. myrt.* "Andr." 2 on NH_4NO_3 medium for 9 days on *Raphanus* leaf discs. Solvent S. Ehrlich's reagent.

Rf values	Colour with reagent	Raphanus leaf discs	
		% growth	Dry weight, mg
Control	0	57.8	9.7
0.00–0.11	0	64.5	9.7
0.11–0.22	0	56.2	10.2
0.22–0.33	blue	60.9	10.1
0.33–0.44	0	61.7	10.2
0.44–0.55	blue (weak)	69.8	11.1
0.55–0.66	0	54.5	9.7
0.66–0.77	0	51.4	9.6
0.77–0.88	0	53.6	10.2
0.88–1.00	0	58.1	10.2

chromatograms of various extract fractions. The leaves were measured under magnification and the largest diameter was recorded (Tables 14 and 15). The differences were only small, but it was nevertheless clear that the uppermost blue spot contributed to the effect of increased area of the leaf discs in comparison with the control. The main object of the experiment was to study the effect of this spot. The effect of the lower spot (IAA) was apparently negative (Table 14), presumably because the concentration was too high. The possibility of supra-optimum concentrations is a drawback in all the bioassays in which unknown amounts of auxin are tested.

b. *Extracts of tryptophan media*

When grown on TTP medium, some *Exobasidium* species, including *E. myrt.* "Andr." 2, readily produce various indole compounds in relatively

Table 14. The effect of scraped-off zones from a chromatogram on the diameter of *Raphanus* leaf discs. Ether extract (pH 2.9) of *E. myrt.* "Andr." 2 on NH_4NO_3 medium for 16 days. Solvent S. Ehrlich's reagent.

Rf values	Colour with reagent	Diameter of leaf discs, mm
Control	0	6.40 ± 0.04
0.30	blue	6.34 ± 0.04
0.48	blue	6.54 ± 0.02

Table 15. The effect of scraped-off zones of a chromatogram on the diameter of *Raphanus* leaf discs. Ether extract (pH 8.1) of *E. myrt.* "Andr." 2 on NH_4NO_3 medium for 16 days. Solvent S. Ehrlich's reagent.

Rf values	Colour with reagent	Diameter of leaf discs, mm
Control	0	6.25±0.04
0.12–0.19	pink (weak)	6.22±0.03
0.20–0.26	pink (weak)	6.25±0.02
0.44–0.50	pink (weak)	6.28±0.05
0.51–0.58	blue	6.44±0.06

large amounts. In these experiments the volumes of extract applied to the chromatogram have therefore been as low as 0.01 to 0.1 ml. Even so, the amount of IAA was much too large for the leaves (Table 16). The IAA on the paper chromatogram was mainly in zone 3 (Rf 0.22 to 0.33), as shown by red coloration with Salkowski reagent and by the coleoptile test. The coleoptile test also showed that there was some IAA in the next zone down, and that there was a zone with significant auxin activity higher up on the chromatogram. The leaves also had a definite positive reaction to this latter zone, which probably contained the same substance as that which had a positive effect from NH_4NO_3 medium. This substance was not coloured by the reagent.

Table 16. The effect of pieces of a paper chromatogram with 0.025 ml extract (pH 2.9) of *E. myrt.* "Andr." 2 on TTP medium (7 days) on the growth of *Raphanus* leaf discs and wheat coleoptiles. Solvent isopropanol : NH_3 : H_2O 10 : 1 : 1. Salkowski reagent.

		Raphanus leaf discs		
Rf values	Colour with reagent	% growth	Dry weight, mg	Coleoptiles mean length, mm
Control	0	44.8	7.5	11.6
0.00–0.11	0	47.5	7.1	11.7
0.11–0.22	0	46.0	7.6	14.5
0.22–0.33	red	38.2	6.7	14.7
0.33–0.44	0	48.5	7.5	11.3
0.44–0.55	0	47.7	7.5	11.4
0.55–0.66	0	49.6	7.3	11.4
0.66–0.77	0	48.3	7.4	11.2
0.77–0.88	0	54.1	8.1	11.7
0.88–1.00	0	54.4	7.6	13.2

Table 17. The effect of pieces of a paper chromatogram with 0.01 ml extract (pH 2.9) of *E. Karst.* 3 on TTP medium (7 days) on the growth of *Raphanus* leaf discs and wheat coleoptiles. Solvent isopropanol : NH_3 : H_2O 10 : 1 : 1. Salkowski reagent.

		Raphanus leaf discs		
Rf values	Colour with reagent	% growth	Dry weight, mg	Coleoptiles mean length, mm
Control	0	32.6	7.4	10.8
0.00–0.11	0	30.9	7.0	11.0
0.11–0.22	0	30.6	6.8	11.2
0.22–0.33	red (weak)	37.4	7.2	14.8
0.33–0.44	red	34.3	7.0	15.2
0.44–0.55	red (weak)	37.9	7.4	11.4
0.55–0.66	0	28.0	6.8	11.0
0.66–0.77	0	32.6	7.3	11.0
0.77–0.88	0	38.2	7.5	14.2
0.88–1.00	0	32.6	7.2	11.5

The coleoptile test revealed the same two active zones in the chromatogram of extract from *E. Karst.* 3 (Table 17). The amounts of extract applied were less in this experiment and the IAA zone also had some leaf-stimulatory effect. The substance with a higher Rf value also had a positive effect, and

Table 18. The effect of pieces of a paper chromatogram of the neutral fraction of ether extract of *E. Karst.* 1 on TTP medium (10 days) on the growth of *Raphanus* leaf discs and wheat coleoptiles. Solvent isopropanol : NH_3 : H_2O 10 : 1 : 1. Salkowski reagent.

		Raphanus leaf discs		
Rf values	Colour with reagent	% growth	Dry weight, mg	Coleoptiles mean length, mm
Control	0	41.3	8.3	8.2
0.00–0.11	0	39.5	8.4	9.0
0.11–0.22	0	38.1	8.0	9.0
0.22–0.33	0	39.2	8.0	8.3
0.33–0.44	0	40.2	7.9	7.8
0.44–0.55	0	34.8	7.5	8.8
0.55–0.66	0	35.5	8.0	9.4
0.66–0.77	0	40.2	8.0	9.3
0.77–0.88	0	48.1	9.2	9.8
0.88–1.00	0	45.6	8.3	9.4

Table 19. The effect on *Raphanus* leaf discs of scraped-off zones of a chromatogram of ether extract (pH 2.9) of *E. myrt.* "Andr." 2 on tryptamine medium (10 days). Solvent S. Ehrlich's reagent.

Rf values	Colour with reagent	Leaf discs % growth
Control	0	48.1
Extr. at pH 2.9		
0.00–0.11	0	48.6
0.11–0.22	0	57.2
0.22–0.33	blue	49.5
0.33–0.44	blue-violet	64.8
0.44–0.55	0	53.3
0.55–0.66	0	54.3
0.66–0.77	0	46.7
0.77–0.88	0	54.3
0.88–1.00	0	51.9
Neutral fract.		
0.22–0.26	blue	47.6

the maximum leaf growth was caused by this zone. The mid-point of the IAA spot lay between Rf 0.33 and 0.44, as shown by the colour reaction and the coleoptile test, and also by measurements of the UV spectrum. However, the spot also graded into the adjacent zones, where the lower concentrations more favourable to growth obtained. The spots were diffuse because the chromatography was on paper. This is one of the major disadvantages of the method. If only one test method is employed, and no comparisons with results obtained from other methods are made, there is a considerable risk that the results may be incorrectly interpreted.

Table 18 shows what happened when all the acid compounds were removed from the extract and the neutral fraction was chromatographed. No clear colour reaction was obtained with Salkowski reagent. The coleoptile test showed that the auxin activity in the IAA zone had disappeared, whereas the other zone was still active. This zone must therefore contain a neutral compound with an auxin effect. This was confirmed by subsequent more detailed investigations. In this experiment the substance also brought about a definite increase in leaf growth. When the neutral fraction was rechromatographed and the zone Rf 0.77 to 0.88 was tested again, the same results as in Table 18 were obtained.

c. *Extracts of tryptamine media*

Ether extracts of *E. myrt.* "Andr." 2 grown on tryptamine medium were chromatographed and tested using *Raphanus* leaves. Maximum leaf growth was caused by the IAA zone (Table 19). It later became apparent that this species is one of the few that produces IAA when grown on this nitrogen source. The effect of the other blue spot further down on the chromatogram is much more doubtful, as is shown by the result of rechromatography of the neutral fraction. The substance, which was shown by fractionation to belong to the neutral fraction, was later identified as tryptophol, an indole compound without much auxin activity. It was difficult to determine whether the other active substance, mentioned earlier, was present in this sample. There was at any rate no coloration in response to Salkowski reagent.

V. STUDIES OF THE AUXIN METABOLISM IN *EXOBASIDIUM*

A. Auxin metabolism on NH_4NO_3 medium

a. *Introduction*

Nielsen in 1930 discovered the so-called rhizopine in the mycelium of *Rhizopus suinus* and *Absidia ramosa*. The substance was purified and identified as IAA by Thimann in 1935. This substance has since then been demonstrated in a number of fungi, using more and more sensitive methods, but these fungi have usually been grown on media containing organic nitrogen sources such as malt extract, potato dextrose agar etc. It has later become apparent that only small quantities of auxin are formed in media containing only glucose and salts, and that the quantities are increased significantly in the presence of peptones and a number of amino acids, particularly tryptophan (TTP). This was found by Brian (1957), for example, in an investigation of the ability of about 25 fungi to synthesize IAA on various nitrogen sources. Sommer (1960) observed weak auxin activity in most, but not all of the filtrates from cultures of 20 fungi (*Basidiomycetes, Ascomycetes* and higher *Phycomycetes*) grown on TTP-free medium. Göttgens (1959) was unable to demonstrate IAA synthesis when *Exobasidium azaleae* was grown on White's medium, and Wolf and Wolf (1952) could detect IAA only when *Exobasidium camelliae* var. '*gracilis*' grew on TTP but not on KNO_3. However, Wolf and Wolf used the Salkowski test (Gordon–Weber's method) which would scarcely show the small amounts of IAA which might be synthesized on this substrate. *Fusarium oxysporum* f. *lycopersici* has been shown to lack the ability to synthesize IAA on all the nitrogen sources tested (Mahadevan and Caroselli, 1964), although the filtrate has been shown to have auxin activity. Ulrich (1960) could classify the mycorrhizal fungi she studied into three groups, according to their ability to synthesize auxins. Group 1 included all *Boletus sp.* except *B. badius,* which could produce IAA even without TTP, Group 2, *B. badius,* which could do this in short experiments but not in long ones; and Group 3, represented by *Amanita,* which could produce IAA + a number of other unidentified indole compounds. In two recent investigations using *Phycomyces Blakesleeanus* with asparagine as nitrogen

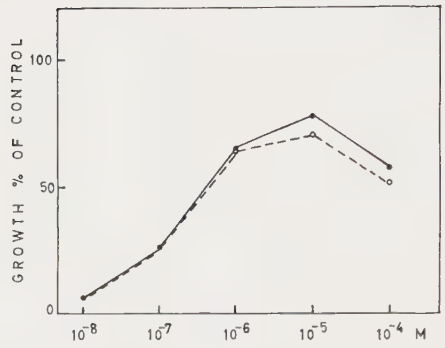


Fig. 5. The response of coleoptiles to IAA (unbroken line) and IAEt (broken line).

source, IAA has also been demonstrated. Besides IAA, Gruen (1965) has also found a substance suspected to be ICA, and some unidentified spots with high Rf values. Halbsguth and Hilgenberg (1965) have found bound TTP in the *Phycomyces* mycelium, besides free and bound IAA. In addition to IAA, Schwinn (1964) found three other substances which were positive in Ehrlich's and Salkowski tests, as yet unidentified, with asparagine as nitrogen source. When I have added asparagine to the medium the results have been mainly similar, both qualitatively and quantitatively, to when a nitrate source has been used.

In growing the various *Exobasidium* cultures, the nutrient solution with NH_4NO_3 as nitrogen source has served as the basic substrate throughout the period of the investigation. Therefore most of the ether extracts studied have been from that medium, but similar results can be obtained with other inorganic nitrate sources, as shown, for example, by experiments with KNO_3. First, the results from the bioassays will illustrate the various active zones which were found on the chromatograms.

b. *Wheat coleoptile tests*

There is no doubt that the wheat coleoptile test is the most sensitive of the bioassays I used, in its ability to respond to different auxins. As shown by the standard curve (Fig. 5) the maximum response of the coleoptiles is to an IAA concentration of 10^{-5} M. The standard error of the mean, expressed in % of the growth of the control, has very seldom exceeded 2% and has usually been much lower than this figure, both in the wheat coleoptile test and the wheat leaf test. The thin layer chromatograms used in the investigations have either been divided up into portions of equal lengths, or else specially interesting zones, shown by spraying replicate spots, have been

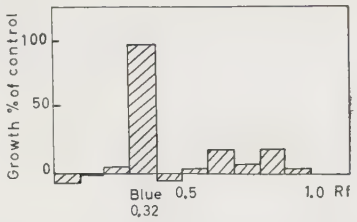


Fig. 6. The response of coleoptiles to the acid fraction of ethyl acetate extract of *E. myrt.* "Andr." 2 on NH_4NO_3 medium (17 days). Solvent S. Ehrlich's reagent.

selected. Equal sized portions of the chromatogram, taken from a part where the solvent has passed, have always been used as controls. It can be difficult to get rid of all traces of this liquid, which may have an inhibiting effect on growth. This is especially true of solvents containing chloroform. Collet (1964) has, on the other hand, shown that the absorption substance itself (Kiselgel G, Merck) can have a positive effect on coleoptile growth at certain IAA concentrations, with a maximum at 10 μg IAA/ml. This may be due to the Ca^{++} ions it contains.

When extract from *E. myrt.* "Andr." 2 at pH 2.9, containing both acid and neutral substances, was used in the test, two active zones giving growth increases were obtained. If the acid and neutral fractions are separated it is clear that one of the zones is due to an acid substance and the other to a neutral. In the experiment shown in Fig. 6, the acid fraction was tested, and, as is apparent in the diagram, there was a very clear peak at an Rf value of 0.32. Spraying with Ehrlich's reagent gave a clear blue-violet colour. There is no doubt that the substance which causes coleoptile growth more than 100% greater than the control in many of these experiments is IAA. Further criteria will be given later.

If the neutral fraction from *E. myrt.* "Andr." 2, is used instead it is apparent (Fig. 7) that IAA is absent from the extract, and the large effect

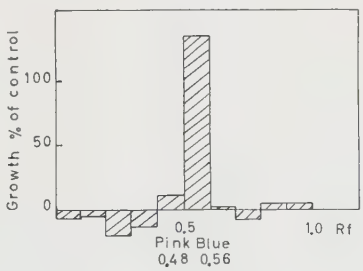


Fig. 7. The response of coleoptiles to the neutral fraction of ether extract of *E. myrt.* "Andr." 2 on NH_4NO_3 medium (10 days). Solvent S. Ehrlich's reagent.

Table 20. The effect on coleoptiles of scraped-off parts of chromatograms of ether extracts (pH 2.9) of fungi grown on NH_4NO_3 medium (9 days). Solvent S. Ehrlich's reagent.

Fungus	Rf value	Colour with reagent	Growth % of control
E. myrt. "Andr." 2	0.11–0.19	brown (weak)	+ 4
	0.28–0.35	blue	+71
	0.40–0.47	0	+ 6
E. vacc. "vit. id." 3	0.05–0.12	violet (weak)	−15
	0.31–0.37	blue (weak)	0
	0.45–0.54	0	− 9

the other active substance has on growth shows clearly. In many cases, a higher growth value is obtained for this substance than for IAA. Ehrlich's reagent gives a pure blue colour with this substance, not quite the same as the more red-violet colour obtained with indoleacetonitrile (IAN). For several reasons, the substance was first identified as IAN and it was only when suitable neutral solvents were used in the chromatography that it became clear that it must be IAA ethyl ester (IAEt). In these solvents it is possible to obtain small but clear differences between the Rf values of the two auxins, as will be shown later. The standard curve for IAEt in Fig. 5 also indicates a maximum response of the coleoptiles to a concentration of 10^{-5} M.

Apart from IAEt, the weak pink spots directly under those in the neutral fraction often have a slight auxin activity.

Using NH_4NO_3 as nitrogen source it was already clear that there were differences between the *Exobasidium* species in ability to produce auxins.

Table 21. The effect on coleoptiles of parts of chromatograms of neutral fractions of extracts of fungi grown on NH_4NO_3 (34 days). Solvent S. Ehrlich's reagent.

Fungus	Rf values	Colour with reagent	Growth % of control
E. vacc. "vit. id." 3	0.50–0.60	0	− 3
	0.60–0.70	0	− 6
E. vacc. "ulig." 3	0.50–0.60	pink (weak)	+ 4
	0.60–0.70	pink	− 3
E. myrt. "vit. id." 2	0.50–0.60	blue	+43
	0.60–0.70	0	−12

Table 22. The effect of parts of chromatograms of extracts (pH 2.9) of *E. myrt.* "Andr." 2 on NH_4NO_3 medium (12 days) on the growth of wheat leaf sections and the diameter of *Phaseolus* leaf discs. Solvent S. Ehrlich's reagent.

Rf values	Colour with reagent	Wheat leaf sections Growth % of control	*Phaseolus* leaf discs Growth % of control
0.28–0.34	blue-violet	+ 17	+ 10
0.46–0.52	pink	+ 7	+ 1
0.53–0.59	blue	+ 19	+ 22

Table 20 refers to scrapings from two chromatograms to which had been applied equal volumes of extract from *E. myrt.* "Andr." 2 and *E. vacc.* "vit. id." 3. Although a weak blue coloration with Ehrlich's reagent showed that small amounts of IAA are also synthesized by *E. vacc.* "vit. id." 3, no elongation of coleoptiles was caused by that zone, whereas the systemic species caused growth 71% over the control. The same low IAA production could be seen in other experiments, in which 0.5–1.0 l of filtrate from *E. vacc.* "vit. id." 3 was extracted and subjected to the coleoptile test.

There was an equally clear difference in the neutral fraction, where in *E. vaccinii* cultures, for example, no trace of IAEt could be detected. The coleoptile test (Table 21) gave the same result, i.e. no perceptible increase in growth. Nor have the weak pink spots any effect. However, extract from *E. myrt.* "vit. id." 2, which attacks the same host but belongs to the systemic group, gave increased growth in the IAEt zone, even though this species has relatively slow auxin metabolism.

c. *Wheat leaf tests*

This method is mainly intended as a gibberellin test. Because of the lack of good leaf tests and the ease of measurement of growth in wheat leaves it has also been used in the auxin investigations, where it has proved useful. The leaves react positively to IAA and IAEt among other things. Both these substances gave maximum growth at 10^{-5} M. Table 22 shows two equal-sized peaks for IAA and IAEt. Growth in test dishes with IAEt varied in different experiments between 11% and 48% of the control, because different concentrations were attained. The pink spot under the IAEt also had a weak effect on wheat leaves (Fig. 8).

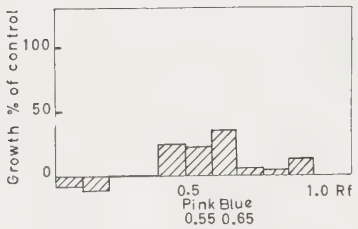


Fig. 8. The response of wheat leaf sections to the neutral fraction of ether extract of *E. myrt.* "Andr." 2 on NH_4NO_3 medium (20 days). Solvent S. Ehrlich's reagent.

d. *Phaseolus tests*

This test, which according to Wheeler (1960) primarily reacts to gibberellins and kinins by leaf area increase, has been used in about 70 experiments to test chromatographed extracts from NH_4NO_3 media. The effect of IAA on the leaf is restricted to causing elongation of the veins, while the mesophyll in between is unaffected. Nor have the experiments shown any effect of IAA, since leaf diameter was measured at right angles to the main vein.

Dale (1966) has recently demonstrated an increase of both fresh weight and diameter of *Phaseolus* leaves with IAA as well as with other growth substances. However, the leaves were weakly illuminated during the experiments, so that the conditions were more like those I used for *Raphanus* leaves.

IAN was the only other indole compound tested (IAEt was not included) which caused larger leaf areas, with a maximum value of 20% over the control, at concentrations of 10^{-5} M. Although IAN has not definitely been demonstrated in *Exobasidium,* it has a biological effect which is broadly comparable with that of IAEt.

Examination of the experiments using the acid fractions reveals that zones causing consistent growth stimulation could be detected only with difficulty. However, the region between Rf 0.80 and 0.90, in chromatograms in Solvent S, often gave an obvious peak of growth.

In contrast, with the neutral fractions it was possible to demonstrate an effect in those cases where there was an IAEt spot, i.e. only when extracts from *Exobasidium* strains which synthesize this compound were used. Increase in growth was again about 20% higher than in the control (Table 22).

e. *Chromatography data*

Chromatography has been used not only in connection with the various bioassays, but also to identify, if possible, the various substances synthesized

Table 23. Ehrlich-sensitive compounds synthesized by *E. myrt.* "Andr." 2 on NH_4NO_3 medium. Rf values are for Solvent S. w = weak.

Acid ether fraction			Neutral ether fraction		
Rf value	Colour	Probable subst.	Rf value	Colour	Probable subst.
0.00	red-lilac				
0.06	blue (w)		0.08	blue (w)	
0.12	pink (w)		0.12	pink (w)	
0.15	yellow (w)	OH-An. A?			
0.20	blue (w)		0.21	blue	tryptophol?
0.25	yellow (w)				
0.32	blue-lilac	IAA	0.32	pink	IAA (trace)
0.41	yellow	An. A			
0.45	pink (band)		0.48	pink	
0.52	blue (w)	IAEt (trace)	*0.53*	blue	IAEt
0.85	pink (w)		0.90	pink (w)	

by the fungi. To make this possible it was necessary to obtain certain reference substances. I succeeded in getting quite a number, but unfortunately there are still some which have been unobtainable. Using the reference substances, mostly various indole derivatives, the reference system (pp. 116–117) in different solvents was established. Most Rf values are means for many chromatograms. The values are in good agreement with those available in the literature, and where differences exist they are almost always such that my Rf values are somewhat higher. In the table there is also information about the approximate chromatography time and the colour reaction of the substances to suitable reagents. The lower limit for detection of these indole compounds is on average at 0.1 μg, when they are sprayed with Ehrlich's reagent and oxidized with nitromuriatic acid vapour, and when a path length of 10 cm is used (Stahl and Kaldewey, 1961).

The Tables 23 and 24 show the Rf values and spot colours which were usually obtained when NH_4NO_3 was the substrate. The extracts come from fungal cultures of various ages so that some spots, particularly the weaker ones, are not visible on chromatograms from the youngest cultures. As in similar tables, the Rf values for the clearest spots have been printed in italics.

These tables (23 and 24) show clearly that *Exobasidium* cultures form many substances which give a colour reaction, even on NH_4NO_3 as nitrogen source. However, most are present only in very small quantities so that it is usually impossible to rechromatograph them in another solvent. Many of the spots show much more clearly when the fungi are grown on a tryptophan medium. Some of the weaker spots are only traces of substances which really

Table 24. Ehrlich-sensitive compounds synthesized by *E. vacc.* "vit. id." 3 on NH_4NO_3 medium. Solvent S.

Acid ether fraction			Neutral ether fraction		
Rf value	Colour	Probable subst.	Rf value	Colour	Probable subst.
0.00	red-lilac				
0.04	blue (w)				
0.12	pink (w)				
0.15	yellow	OH-An. A?			
0.17	blue (w)				
0.25	yellow (w)				
0.30	pink	ICA			
0.34	blue-lilac	IAA	0.33	pink (w)	IAA (trace)
0.43	yellow	An. A			
0.50	pink (w)		0.50	pink (w)	

belong to the other fraction. This is especially true for neutral compounds which are present in high concentrations.

E. myrt. "Andr." 2 forms two indole compounds in large amounts, the two which were earlier shown to have an auxin effect in bioassays, viz. IAA and IAEt. Although the IAA spot is very clear, it has not been possible to get clear UV curves of this labile substance when the fungus is grown on NH_4NO_3 medium. However, this has succeeded with IAEt spots and its UV spectrum is shown in Fig. 9. Comparison of this curve with that for synthetic IAEt (Fig. 16) reveals very great similarities. Both curves have absorption peaks at 272, 282 and 292 nm. The curve obtained from the same part of a chromatogram with *E. vacc.* "vit. id." 3, with no blue spot, as usual, has only slight similarities with that of the reference substance. The IAEt spot is also absent from Table 24, even though this is an amalgamation of results from about a hundred chromatograms. Very few neutral compounds are formed by *E. vaccinii* cultures.

However, the acid fractions contain the same substances, in the main, with the exception that indolecarboxylic acid (ICA) is found in *E. vacc.* "vit. id." 3, which is of interest, as will be seen later. In both *Exobasidium* species there are clear yellow spots with Ehrlich's reagent at about Rf 0.41, and there is no doubt that it is anthranilic acid (An.A) which causes these. This substance gives an intense blue fluorescence when illuminated with an UV lamp, and the Rf values for the spots from the extract and the reference substance have always agreed, irrespective of the solvent used. In addition, the UV spectrum of the spot is very similar to that of the An.A, but it is completely different from that of indole compounds (Fig. 10).

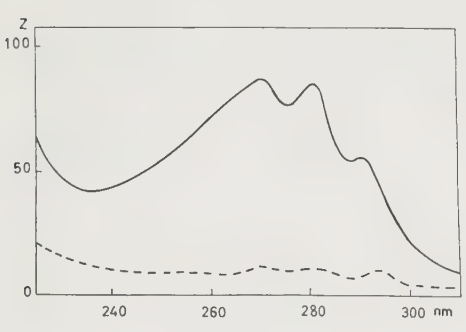


Fig. 9.

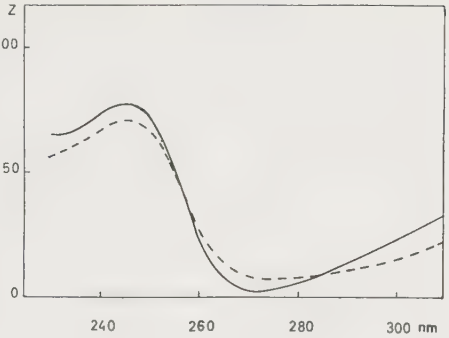


Fig. 10.

Fig. 9. UV absorption spectra of region Rf 0.42–0.54 of chromatograms (Solvent S) of the neutral ether fractions of *E. myrt.* "Andr." 2 (unbroken line) and *E. vacc.* "vit. id." 3 (broken line) on NH_4NO_3 medium (14 days). There was a blue spot with Ehrlich's reagent for *E. myrt.* "Andr." 2 in this zone.

Fig. 10. UV absorption spectrum of region Rf 0.42–0.48 of a chromatogram (Solvent S) of the neutral fraction of *E. vacc.* "vit. id." 3 on NH_4NO_3 medium for 12 days (unbroken line), compared with spectrum of An. A (broken line).

A mysterious substance which has appeared only once has not been included in Tables 23 and 24. The spots have been very pronounced and have been mainly from the neutral fraction, at Rf 0.90. The substance was first coloured yellow by spraying with Ehrlich's reagent, but after only a short time in daylight the colour changed so that the spot became blue-green instead. It has later become clear that the substance was diphenylamine, which had been added to the ether (a new manufacture, not used since) as a preserative in very small amounts, but which had been concentrated during the evaporation.

f. *Synthesis of tryptophan*

It can be said that, compared with other species of fungi, most *Exobasidium* species readily synthesize IAA on an inorganic nitrogen source, and a number of other indole compounds can be detected in the culture medium. However, in this connection it should be stated that thin-layer chromatography very greatly have improved the possibility of detecting this type of substance.

It is now fairly generally believed that IAA synthesis also occurs via TTP in an inorganic medium, since this amino acid can be found in such media, but this does not exclude the possibility of other pathways to IAA. I have myself attempted to detect TTP in fungal cultures of different ages, an attempt in which certain difficulties arose because TTP is not soluble in ether or comparable extractants and extraction with butanol gave no certain

Table 25. Ehrlich-sensitive compounds synthesized by *E. myrt.* "Andr." 2 (A) and *E. vacc.* "vit. id." 3 (B) on anthranilic acid medium. Solvent S.

A.				*B.*			
Acid		Neutral		Acid		Neutral fraction	
Rf value	Colour	Rf value	Colour	Rf value	Colour	Rf value	Colour
0.00	lilac			0.00	lilac	No spots	
0.32	blue-lilac			0.32	blue-lilac		
0.40	yellow (definite)	0.53	blue	*0.40*	yellow (weaker)		

answer. If the aqueous phase is evaporated off after the ether extraction, the residue is a viscous and sticky mass, consisting of sugars, salts etc., which cannot be chromatographed. However, I have added to this a small volume of hot methanol, in which TTP is moderately soluble while only small quantities of sugars dissolve in it. Small volumes of the methanol could be applied to the chromatogram. The spots which showed after spraying with ninhydrin reagent had Rf 0.00 in Solvent S, and 0.05 in the more polar Solvent Sp, which are both identical with the TTP reference chromatographed at the same time. However, the amounts of the substance were not large, which is probably because TTP almost immediately reacts further. The clearest TTP spots were obtained when the later steps in the pathway of IAA synthesis were blocked with dimedone causing an accumulation of those metabolites, including TTP, before the blocked step.

The biosynthesis of TTP is considered by Gordon (1961) and Karlson (1961), among others, to take place as follows:

$$\rightarrow \text{anthranilic acid} \rightarrow \text{indole} \rightarrow \text{tryptophan}$$
$$\text{serine}$$

The indole aromatic ring and the N atom come from the anthranilic acid, which in fungi probably arises from chlorismic acid in only one reaction step (Lingens, Sprössler and Goebel, 1966). The indole and serine then condense to form tryptophan. This chain of synthesis seems very probable in *Exobasidium.* As shown by Tables 23 and 24, An.A has been found both in *E. myrt.* "Andr." 2 and *E. vacc.* "vit. id." 3, and also in the other species I have cultured. Also, this is the very first substance which can clearly be shown after inoculation, which indicates that it is one of the first reaction products in the metabolism. When *E. myrt.* "Andr. " 2 and *E. vacc.* "vit. id." 3 were grown on An.A as the only nitrogen source the following result was obtained (Table 25).

Thus these species can synthesize IAA (0.32) from An.A (0.40), and *E. myrt.* "Andr." 2 also forms IAEt (0.53). That it was in fact this latter compound was substantiated by a coleoptile test in which the coleoptiles in the test dishes showed a growth increase 50% more than the control. Table 25 also shows that *E. vacc.* "vit. id." 3 converted a much larger proportion of the An.A than the other species. This did not, however, result in greater production of IAA.

The next metabolite in TTP synthesis, i.e. indole, could not be clearly demonstrated, at any rate not in young cultures. However, in old cultures weak spots which might correspond to this substance were obtained, but this is probably a result of breakdown of IAA rather than a step on the way to TTP, unless it can be assumed that the indole formed as a breakdown product rapidly takes part in TTP synthesis again.

Gibson *et al.* (1956) have shown that indole synthesis in *Escherichia coli* is greatly stimulated by magnesium ions. An experiment was carried out with or without additions of $MgSO_4$ (10^{-3} M) and serine (10^{-2} M). If without additions, then the $MgCl_2$ which is normally a constituent of the fungal medium was omitted. Then the amount of IAA synthesized was scarcely detectable, whereas it was very clear when the additions had been made. However, the result must chiefly be due to the fact that growth on the medium without Mg^{++} ions was much less than it otherwise would be.

B. Auxin metabolism on N–Z case, casamino acids, yeast extract and maize leaf extract

The ability of several *Exobasidium* species to synthesize auxins on more complex organic nitrogen sources has been tested. The first three chemicals listed in the title are commonly used in the Institute, the maize leaf extract I have prepared myself. Young maize leaves have been cut into small pieces and ground in a mortar with water, ice and fine sand as an abrasive. Maize leaves were chosen because they happened to be available at the time. After centrifuging, the filtrate was autoclaved and 10 ml was added to flasks containing 90 ml of Medium 1 with no nitrogen source. Fungal growth was also good on this medium. The reason for its use was the advisability of growing the fungi on a substrate perhaps more similar to that encountered when they grow on leaves, to see what auxins they formed.

Results obtained on N-Z case, casamino acids and yeast extract were broadly comparable, and Table 26, can therefore be taken as representative for all these substrates. Only the acid fraction has been included in this table, when no obvious spots were observed in the neutral one.

Table 26. Ehrlich-sensitive compounds synthesized by various strains of *Exobasidium* on N-Z case medium. Solvent S.

E. myrt. "Andr." 2				*E. vacc.* "vit. id." 3				*E. Karst.* 3		*E. jap.* 1		*E. vacc.* "Arct." 2	
Acid		Neutral		Acid		Neutral		Acid		Acid		Acid fraction	
Rf	Col.	Rf	Col.	Rf	Col.	Rf	Col.	Rf	Col.	Rf	Col.	Rf	Col.
0.03	blue	0.24	blue (w)					0.00	blue (w)	0.00	lilac (w)		
0.28	pink	0.28	pink					*0.28*	pink	0.14	blue	*0.28*	pink
0.32	blue	0.32	blue (w)	*0.32*	blue			*0.32*	blue	*0.32*	blue	*0.32*	blue
0.45	pink	*0.45*	pink										
0.53	blue (w)	*0.53*	blue			0.53	blue (w)						

There are three main differences between Table 26 and that for NH_4NO_3 as nitrogen source. The first is concerned with a substance which is absent here, i.e. An.A. It has not been possible to find this substance—a further indication that An.A is not a breakdown product but an important step in the synthesis of IAA from an inorganic nitrogen source. The second difference is quantitative, and concerns the pink spot in the acid fraction with Rf 0.28, i.e., ICA. This substance is found in most fungi and often in fairly large amounts. The synthesis of ICA in large amounts is connected with the fact that IAA is produced more readily than on NH_4NO_3, as will be shown later. It has therefore also been possible to get a fairly clear UV spectrum for the IAA spot (Rf 0.32). The curve has a minimum at 250 nm and maxima at 280 (the highest) and 290 nm, and closely follows that of the reference substance. Coleoptiles also reacted positively (+85%) to the IAA zone of a chromatogram of the acid ether fraction.

The third difference is in the blue spot at Rf 0.53 in the neutral fraction. This is very obvious in *E. myrt.* "Andr." 2, and had an elongating effect on the coleoptiles, the increase in length of which was 80% more than the controls (Fig. 11), about the same as was obtained with the IAA spot in the previous experiment. There is no question that the substance is IAEt and the wide range of concentration over which it is active is also illustrated by Fig. 11. Maximum elongation was obtained at a dilution of 1/25 of the original concentration.

It is not so remarkable that IAEt has been found in *E. myrt.* "Andr." 2, as that it has also been demonstrated in *E. vacc.* "vit. id." 3, on two occasions on N-Z case medium. The intensity of the colour of the spot was low but the sensitive coleoptiles reacted positively to such an extent (71%) that there

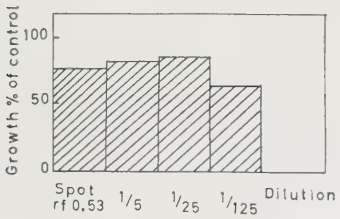


Fig. 11.

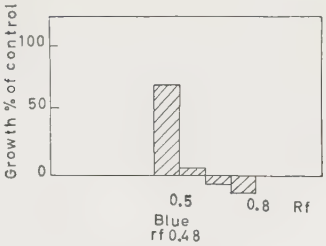


Fig. 12.

Fig. 11. The response of coleoptiles to various concentrations of the blue (Ehrlich's reagent) spot at Rf 0.53. Solvent S. Neutral fraction of ether extract of *E. myrt.* "Andr." 2 on N–Z case (10 days).

Fig. 12. The response of coleoptiles to basic (pH 8.1) ether extract of *E. vacc.* "vit. id." 3 on N–Z case medium (16 days). Solvent S. Ehrlich's reagent.

is little doubt that the auxin is also present in this species (Fig. 12). However, the small quantities of auxin have not allowed rechromatography and UV measurement, so the identification is not completely certain.

It is even more difficult to determine the identity of the other weakly coloured spots with no auxin activity in Table 26. These are not discussed here but some of them will be included in the chapter on TTP metabolism.

Chromatography of extracts from fungi which grew on maize leaf extract gave a brown-lilac zone from the beginning to Rf 0.15 and also a number of other spots originating from the maize leaves. Besides these, which had no effect in the bioassays, most of the substances listed in Table 26 could be demonstrated. The blue spot in the neutral fraction from *E. vacc.* "vit. id." 3, mentioned earlier, was also present. Maize leaf extract and N-Z case have up to now appeared to be the only substrates on which representatives of *E. vaccinii* and other forms causing localized spots on the leaves of their

Table 27. The effect on coleoptiles of parts of chromatograms of ether extracts (pH 2.9) from fungi grown on corn leaf extract (25 days). Solvent S. Ehrlich's reagent.

Fungus	Rf values	Colour with reagent	Growth % of control
E. vacc. "vit. id." 3	0.50–0.60	0	+ 4
	0.60–0.70	blue (w)	+ 70
E. vacc. "vit. id." 1	0.30–0.40	yel.-brown	± 0
	0.40–0.50	blue	+ 121

Table 28. Ehrlich-sensitive compounds synthesized by *E. myrt.* "Andr." 2 on TTP medium. Solvent S.

Acid ether fraction			Neutral ether fraction		
Rf value	Colour	Probable subst.	Rf value	Colour	Probable subst.
0.00	red-lilac				
0.04	blue	ILA?			
0.10	yel.-orange	OH-An. A			
0.12	red-lilac		0.12	blue	IAM?
0.21	yellow (w)		*0.21*	pinkish-blue	IAld
0.28	pink	ICA	0.28	pink (w)	ICA (trace)
0.32	blue-lilac	IAA	0.32	blue (w)	IAA (trace)
0.42	yellow	An. A	0.42	yellow (w)	An. A (trace)
0.45	pink		*0.45*	pink	IAAld?
0.52	blue (w)	IAEt (trace)	*0.52*	blue	IAEt
			0.74	brown-lilac	Indole

hosts were able to form this auxin (IAEt), if the cases where IAA was added to the medium are excepted. Results of two coleoptile tests with this substance (IAEt), produced by *E. vacc.* "vit. id." 1 and 3, show (Table 27) that increases in growth can be more than 100%.

C. Auxin metabolism on tryptophan medium

a. *Results of long term experiments*

It is now generally thought that TTP (L form) is one of the most important indole derivatives in plants, partly because it is a precursor of IAA. TTP has been demonstrated in plants in both free and bound forms. The largest quantities are concentrated in the shoot and root meristems (Stowe, 1959), parts of the plant where IAA synthesis is intense. From the beginning of my investigations, I have therefore used TTP in the medium and it was immediately apparent that a number of substances were synthesized in the presence of various *Exobasidium* species.

There were two types of experiments with TTP; long term,—usually more than five days—; and short term,—24 hours or less. Since, in the main, the long term experiments were done first and the extracts from them contained about the same substances from the same fungus, they will be discussed first. The more rapid happenings in the short term experiments will then be followed in greater detail. In Table 28 the clearest and most frequent spots among the many on the chromatograms are listed. *E. myrt.* "Andr." 2 has

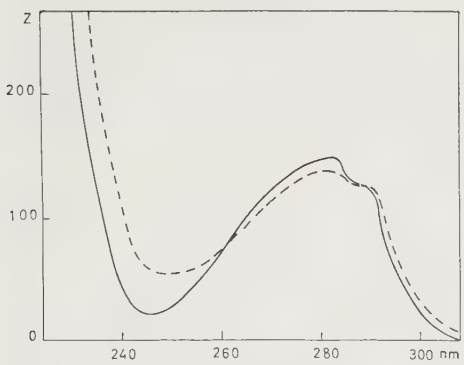


Fig. 13.

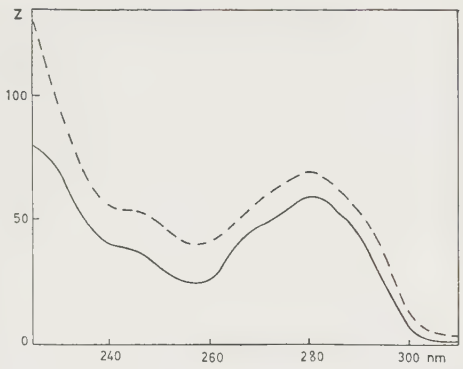


Fig. 14.

Fig. 13. UV absorption spectrum of region Rf 0.02–0.07 (blue with Ehrlich's reagent) of a chromatogram (Solvent S) of the acid fraction of an ether extract of *E. uvae ursi* 2 on TTP medium for 16 days (broken line). The spectrum of indolelactic acid (unbroken line) is shown for comparison.

Fig. 14. UV absorption spectrum of the pink (Ehrlich's reagent) spot at Rf 0.28 (broken line). Acid fraction of an ether extract of *E. myrt.* "Andr." 2 on TTP medium (8 days). Solvent S. The spectrum of ICA (unbroken line) is shown for comparison.

been selected as representative of the fungi. Together with *E. myrt.* "Andr." 1, it has been found to be the greatest producer of auxin in the genus. A corresponding table of values obtained in an alkaline solvent (A) includes the same substances, in the main, plus some weak spots which are probably breakdown products.

Most of these spots are included on Plate 1. The similarities between the extract's spots and the spots for reference substances are clearly shown by the chromatogram.

Discussion of the spots in Table 28 will begin with those of the acid fraction. A fairly weak red-brown spot almost always remains at the starting point. The colour is often seen in the extract even before chromatography and is probably one of the pigments synthesized by many fungi.

The next compound, which is coloured blue by Ehrlich's reagent, is clear especially in extracts from older cultures. In the table the substance has been provisionally named as indolelactic acid (ILA), and its UV spectrum (Fig. 13) is very similar in many respects to that of ILA. However, a similar blue spot with about the same Rf value in S is formed, when the fungus has been incubated for some time with IAA, and this is very probably 5-OH-IAA. The UV curve for this compound is clearly different from the curve in Fig. 13, so that one may deduce that two substances are involved. Neither of them has had any great effect in various auxin tests.

The yellow-brown spot at Rf 0.10 also appears late during incubation with TTP and it is probably OH-anthranilic acid since the Rf values also agree in Solvents A and Sp, and the substance is coloured in a characteristic way. It is previously known as TTP-metabolite and, according to Karlson (1961), has been observed during breakdown of TTP.

tryptophan → Formyl—kynurenine → kynurenine → 3-OH-kynurenine →

→ 3-OH-anthranilic acid + alanine → "amino-aldehyde" → quinolinic acid / nicotinic acid

Two experiments to demonstrate kynurenine and kynurenic acid have not succeeded.

The next clear substance which can be identified is indolecarboxylic acid (ICA), which has also been met with previously in other media. The Rf values have agreed with those of the test substance in all the solvents tried. The neutral no. V, consisting of chloroform: ethanol (65: 35), is especially suitable for separating ICA from IAA. Otherwise these two substances often lie so close together that they fuse into a single spot. ICA and IAld are also coloured by Ehrlich's reagent, but the pink colour develops much more slowly than in other indole compounds, which makes the identifications of these substances still more reliable. To judge from the curves in Fig. 14, there is no doubt either that the substance scraped from the chromatogram is ICA. Besides the absorption peak at 280 nm there is a very characteristic hump in the curve at 245 nm which I have only found for ICA. In contrast to carboxylic acids with several carbon atoms in the side chain, this indole compound has no auxin activity, but this seems to be contradicted by the diagrams from some of the bioassays. However, this effect is certainly because some of the nearby IAA spot has been included. The place of ICA in auxin metabolism is further discussed in the chapter on IAA breakdown (V, L).

Comments about the IAA spot will be made at the end of the section, since more detailed investigations have been made of the synthesis of this important auxin. So the next spot in the acid fraction is the yellow An.A one. This substance has been discussed previously, and the reason that it also appears on TTP medium must be that it also is a breakdown product of TTP. This is also shown by short term experiments. It would be interesting to see whether this An.A undergoes further breakdown, or whether it at once takes part in the synthesis of TTP, described earlier.

The first spot in the neutral fraction is a weak blue one at Rf ca. 0.12, very probably indoleacetamide (IAM), if one decides on the basis of Rf values in other solvents. However, this substance is only found in *Exobasidium* species which produce large amounts of IAA, while in other species it only

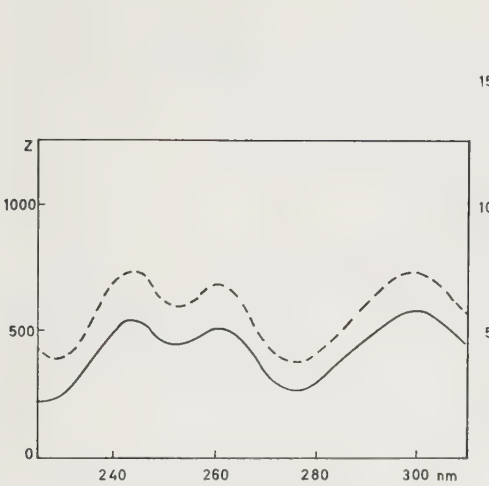


Fig. 15.

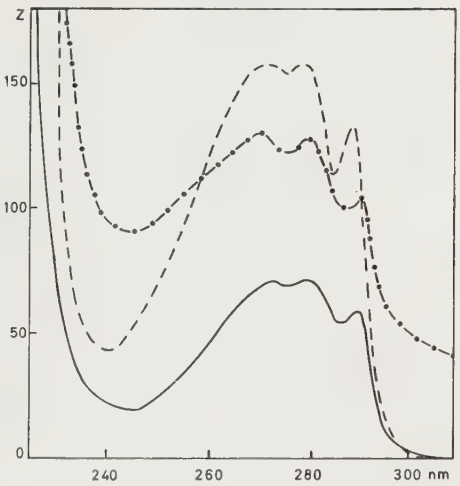


Fig. 16.

Fig. 15. UV absorption spectrum of IAld (unbroken line) compared with that of the pink-blue (Ehrlich's reagent) spot at Rf 0.21 (broken line). Neutral fraction of an ether extract of *E. myrt.* "Andr." 2 on TTP medium (12 days). Solvent S.

Fig. 16. UV absorption spectrum of the blue (Ehrlich's reagent) spot at Rf 0.53 on a chromatogram (Solvent S) with the neutral fraction of an ether extract of *E. myrt.* "Andr." 2 on TTP medium for 10 days (—·—·—). The spectra of IAEt (——) and IAN (– – –) are shown for comparison.

appears when they are incubated with IAA. According to Fawcett (1961), naturally occuring IAM has not been found in plants, but it could be detected when IAA was introduced into the plant tissues. Unfortunately the amount of IAM has been so small that no clear UV spectrum could be obtained.

Although the next spot, at Rf 0.21, is also weak, it has been possible to measure an UV curve, and this is so identical with that of indolealdehyde (IAld) that there can be no doubt that this substance is also synthesized by *Exobasidium*. The curves in Fig. 15 show pronounced maxima at 245, 260 and 295 mm, and no other indole compound has an UV curve with such an appearance. This substance, which is also one of the breakdown products of IAA, has been tested on both wheat and *Phaseolus* leaves. The results of the experiments shown in Table 29, with spots scraped from the chromatogram and known concentrations of IAld, are in complete agreement in as much that there is no stimulatory effect on either of the leaves.

The narrow pink band at Rf 0.45 which first occurs in the neutral fraction, is a very labile compound and therefore difficult to investigate properly. However, the Rf values and the fact that the substance is so easily converted

Table 29. The effect on leaves of the blue (Ehrlich's reagent) spot at Rf 0.21 in the neutral fraction of ether extract of *E. myrt.* "Andr." 2 on TTP medium (37 days). Solvent S. The effect of various concentrations of IAld is shown for comparison.

Test method	Growth % of control with			
	The tested spot	IAld		
		10^{-6} M	10^{-5} M	10^{-4} M
Wheat leaf sections	−3 to −6	−2	−2	+1
Phaseolus leaf discs	−8 to +6	+3	+3	−5

indicate that it may be a question of small quantities of indoleacetaldehyde (IAAld). In a number of the bioassays a positive effect has been obtained from this zone of the chromatogram, depending on conversion of the aldehyde to IAA in the plant tissues. In short term experiments the substance has been shown with more certainty.

The uppermost blue spot in the neutral fraction is often IAEt. This substance, as has been mentioned, was assumed for a long time to be IAN, which is also in the neutral fraction and has about the same Rf value in Solvents S and A. The differences in colour between the spots were put down to concentration differences, since very small quantities of a substance which is normally coloured blue become pink instead. In the neutral Solvent I, the substance agreed much better with IAEt than with IAN. The Rf values are then 0.62 and 0.49, respectively. Neutral extract has also been applied to the starting points of the two compounds, and subsequent chromatography has resulted in two separate spots at the IAN spot, but still only a single spot with IAEt. This is evidence that IAEt must be the compound in question.

Although the IAEt spots are usually clear, and have a strong auxin effect, it has been difficult to get clear UV curves. The curve in Fig. 16 like that drawn earlier for NH_4NO_3 medium in Fig. 9 is therefore an example of one of the few successful attempts to show distinct peaks. In Fig. 16 the UV spectrum has been compared with that of IAN and IAEt. The curves for these substances can be seen to be confusingly alike, yet there are a number of differences. Thus the last two maxima in IAN come 2 nm earlier than in IAEt, i.e. at 278 and 288 nm instead of 280 and 290 nm. The minimum before the last minor peak is also much earlier in IAN. As is shown, the curve for the extract is in complete agreement with that of IAEt in all these details,

Table 30. The growth of wheat leaf sections treated with parts of chromatograms of ethyl acetate extracts (neutral fractions) of *E. myrt.* "Andr." 2 on TTP medium (68 days). Solvent S. Ehrlich's reagent.

Rf values	Colour with reagent	Growth % of control
0.09–0.13	blue	+ 9
0.37–0.42	pink	+ 12
0.44–0.50	blue	+ 28
0.44–0.50 diluted to 1/3		+ 30
0.44–0.50 diluted to 1/9		+ 19
0.44–0.50 diluted to 1/27		+ 15

and also in the position of the first minimum. There is therefore no doubt that it is this substance which is present in the sample.

Experiments to determine the effect of IAEt on leaves have given a result largely similar to the previous ones, i.e. no stimulation in *Phaseolus* leaves, where the result varied from −3 to ±0%, but a small but clear effect can be seen from the results of wheat leaf experiments. This is shown by an experiment (Table 30), in which two of the other spots from the neutral fraction have also been tested, viz. the blue one at Rf 0.12 (IAM) and the pink at Rf 0.40. For these, growth increase was about 10%, whereas at suitable dilutions of IAEt it reached 30%.

Although representatives of most *Exobasidium* species were grown on TTP medium in long term experiments, it was only the species which systemically infect *Andromeda polifolia* which formed clear IAEt spots. These are *E. Karstenii* 1–6 and *E. myrt.* "Andr." 1–2. For *E. vaccinii*-forms there were no

Table 31. The effect on coleoptiles of parts of chromatograms of ether extracts (neutral fractions) of fungi grown on TTP medium (28 days). Solvent S. Ehrlich's reagent.

Fungus	Rf values	Colour with reagent	Growth % of control
E. vacc. "vit. id." 3	0.35–0.45	0	+ 1
	0.45–0.55	0	+ 12
	0.55–0.65	0	+ 6
	0.65–0.75	red-lilac (weak)	± 0
E. uvae ursi 2	0.40–0.50	0	+ 16
	0.50–0.60	blue (weak)	+ 45

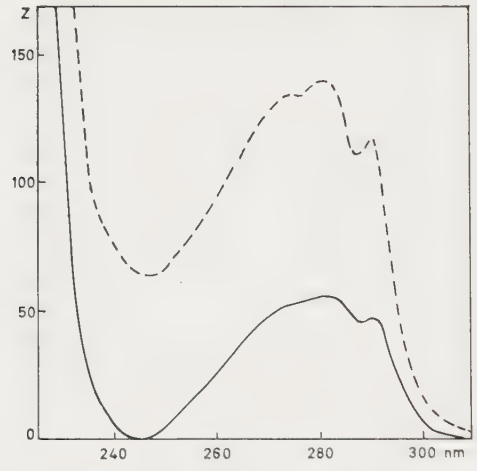


Fig. 17.

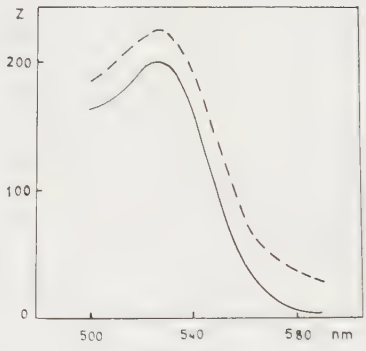


Fig. 18.

Fig. 17. UV absorption spectra of chromatographed IAA (unbroken line) and the blue-violet (Ehrlich's reagent) spot at Rf 0.32 on a chromatogram (Solvent S) with the acid ether fraction of *E. myrt.* "Andr." 2 on TTP medium for 6 days (broken line).

Fig. 18. Spectrophotometer measurements of the acid ether extract of *E. myrt.* "Andr." 2 (broken line) and IAA (unbroken line) after the addition of Salkowski reagent.

such spots and the coleoptiles have for the most part not reacted positively to those zones of the chromatogram where IAEt would lie. On two occasions, when extraction was from large amounts of filtrate (2–3 litres), a slight stimulation (+12%) in the IAEt region could also be recorded for the latter species but there was no detectable colour with the reagent (Table 31). The same experiment showed that this effect was even clearer for the systemic species *E. uvae ursi* 2, which gave a 45% increase in growth.

A red-lilac spot at 0.65–0.75 from *E. vacc.* "vit. id." 3 was also included in Table 31. It had no auxin effect and was encountered especially in old cultures. The chromatography data indicated that this was indole, but this is not certain, because the amounts were very small and in most solvents the spots lie just behind the front, which makes the Rf values not very reliable.

We now come to the production of IAA on TTP medium. All the evidence from chromatography obtained up to now, with paper chromatography and thin layer chromatography, has shown that IAA is synthesized. The Rf values are in complete agreement with those of synthetic IAA in all the solvents listed on p. 13. Further evidence for the identification is given by the spectrophotometer measurements. The UV curve (Fig. 17) for the scraped-off spot agrees in all details with that of the reference substance. In these meas-

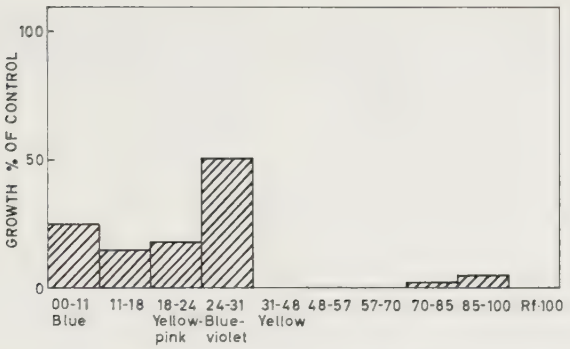


Fig. 19. The response of coleoptiles to parts of a chromatogram (Solvent S) of the acid fraction of an ether extract of *E. myrt.* "Andr." 2 on TTP medium (5 days). Ehrlich's reagent.

urements, as with other similar ones, different concentrations have been chosen, so that the curves would not be too much on top of one another. Such an appearance of the curve is typical for indole derivatives with no double bond in the side-chain connected to the ring system (Kaper and Veldstra, 1958). Spectrophotometric measurements in the range of wavelengths 500–590 nm have also been made after the addition of Salkowski reagent to concentrated amounts of the substance from the IAA zone of the chromatograms. Fig. 18 shows that the curves agree well, and the red colour obtained with the reagent absorbs light most at 525–530 nm both in the tested sample and in IAA.

Fig. 19 gives a fairly representative picture of a coleoptile test of the most important spots from the acid fraction of an extract from a young culture.

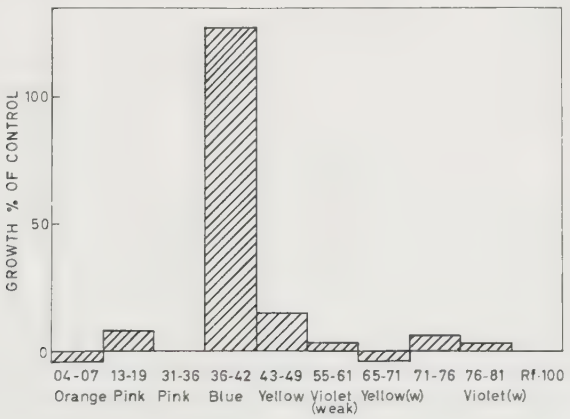


Fig. 20. The response of coleoptiles to parts of a chromatogram (Solvent A) of the acid fraction of an ether extract of *E. myrt.* "Andr." 2 on TTP medium (10 days). Ehrlich's reagent.

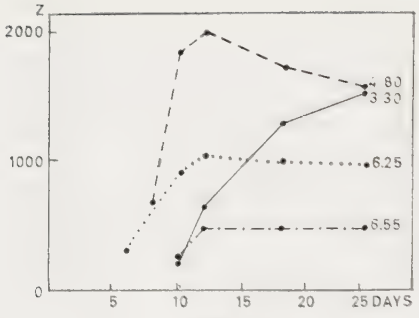


Fig. 21.

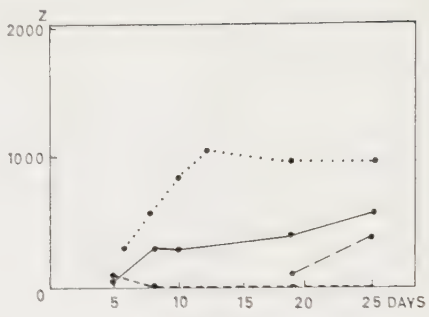


Fig. 22.

Fig. 21. The production of IAA by *E. Karst.* 3 on TTP medium at different pH values. Measured spectrophotometrically at 530 nm after the addition of Salkowski reagent.

Fig. 22. The production of IAA by some strains of *Exobasidium* on TTP medium at pH 6.25. Measured spectrophotometrically at 530 nm after the addition of Salkowski reagent. *E. Karst.* 3 = ·····, *E. Karst.* 6 = ———, *E. vacc.* "oxyc." 3 = — — —, *E. cass.* 1 = – – – –.

IAA showed itself to be already the most active, and in addition there was also some stimulation by the first zones. The weak spots in the first zones scarcely brought about this effect, but it was due to the weak brown trail from Rf 0.00 to 0.15, which can be observed in younger cultures and which is probably indolepyruvic acid (IPyA). This will be discussed in more detail in connection with short term experiments. If chromatography was in Solvent A instead, and the extract was from an older culture, there was only one really active zone, as Fig. 20 shows, namely that due to the presence of IAA.

At an early stage of the investigations, the effect of various environmental factors on the synthesis of IAA from TTP was studied. Flasks containing buffered TTP medium were inoculated in the usual way, and then 1 ml samples of the contents were taken at regular intervals, under sterile conditions. After centrifuging, 2 ml Salkowski reagent was added and the colorimetric values were measured. The curves in Fig. 21 and 22 were drawn from and after the day when a positive deflexion, i.e. red coloration, was first obtained. The effect of pH on IAA synthesis was studied in the first of these experiments. The middle pH values (4.8 and 6.25) favour rapid onset of production and at pH 4.8, in particular, a very large amount of IAA is formed and then gradually decreases. At the highest and lowest pH values it takes longer before a large enough amount is formed for the reagent to give a positive deflexion. After this has happened, the values rise rapidly at pH 3.3, whereas synthesis is inhibited all the time at pH 6.55. These results are correlated with the growth of the fungus, as clearly shown by measurements of the extinction value at 610 nm made at the same time.

Table 32. The IAA production by various strains of *Exobasidium* on TTP medium. Initial pH 4.5. Ether extracts at pH 2.9. The extinction after adding Salkowski reagent was measured at 530 nm and expressed as Z values.

Fungus	Incubation period, days	Final pH	Z-value
E. vacc. "vit. id." 2	1	4.45	0
	2	4.0	0
	4	3.7	0
	7	4.4	0
	10	6.25	0
E. vacc. "oxyc." 1	1	4.5	98
	2	4.2	205
	4	4.1	191
	7	3.5	190
	10	3.35	157
E. myrt. "vit. id." 3	1	4.5	0
	2	4.4	0
	4	4.4	0
	7	3.5	0
	10	5.3	0
E. cass. 2	1	4.4	0
	2	4.25	63
	4	4.0	170
	7	3.6	170
	10	5.0	157

Another factor, which also influences growth, is whether the culture is on a shaker or not. Thus the IAA content in a shaken culture is considerable higher, at first, than in a similar flask which is not shaken. However, after a time the latter attains the same value as the shaken culture. Temperature also affects IAA synthesis in the same way, i.e. it is most rapid at that temperature which is optimum for growth of the species in question.

As already indicated, there seem to be quite large differences between the ability of the various *Exobasidium* species to synthesize IAA. In Fig. 22, four different species have been compared. The two strains of *E. Karstenii* rapidly formed large amounts of IAA, while the first positive result for *E. vacc.* "oxyc." 3 was not obtained before 19 days after inoculation. However, the curve then continued to rise. *E. cass.* 1. gave weak red coloration on the 5th day and still weaker on the 7th, then none at all. Probably breakdown of the auxin occurs as rapidly as synthesis, an idea supported by the fact

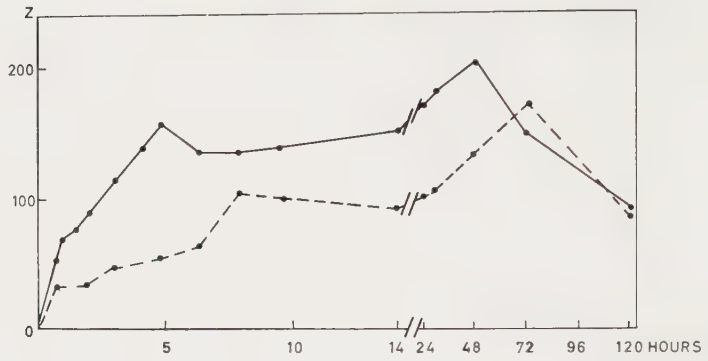


Fig. 23. The production of IAA by *E. myrt.* "Andr." 2 (unbroken line) and *E. vacc.* "vit. id." 17 (broken line) during a short term experiment on TTP medium. Measured spectrophotometrically at 530 nm after the addition of Salkowski reagent.

that there are strong ICA spots from this fungus. However, the fungus also grows somewhat more slowly than the others.

The following investigations of IAA synthesis on TTP medium were done much later and partly in a different way, although still with the addition of Salkowski reagent. In the first experiment 41.4 mg of DL-TTP was dissolved in 100 ml fungal medium, which was then sterilized by filtering and divided into 5 ml portions per flask. Thus each flask began with less than 2 mg TTP. The inoculum which was then put in the flasks was sufficiently large to give a relatively dense fungal suspension from the beginning. After removing 1 ml from the flasks, they were extracted at pH 8.1 and 2.9. Table 32 shows that the final pH for all the species except *E. vacc.* "oxyc." 1 follows the usual pattern for fungi cultivated on a nutrient solution containing NH_4NO_3, i.e. the value first goes down to ca. pH 3.5 and then begins to rise again. Of the tested species, which are all relatively poor IAA-producers, there were only two which could form a sufficiently large quantity of IAA for there to be any colour with the reagent solution. *E. vacc.* "oxyc." 1 produced the largest amounts, and *E. cass.* 2. almost as much. The maximum was reached after 4–7 days, then the amount of IAA decreased, in both, on the 10th day. Not even in chromatograms of extract from *E. vacc.* "vit. id." 2 and *E. myrt.* "vit. id." 3 were there any clear IAA spots.

In the next experiment (Fig. 23) the TTP was added to a phosphate buffer with pH 4.5, so that TTP was the only nitrogen source. Washed conidia from 8 day old cultures of *E. myrt.* "Andr." 2 and *E. vacc.* "vit. id." 17 were then added to the flasks. For an *E. vaccinii*-form, the latter produces a relatively large amount of IAA. After adding the conidia, the density of the suspension was measured at 610 nm and was shown to be the same in both

the flasks, with Z values of 670. Samples were then taken for four days. During this time there was no red colour with the reagent in the control flask without fungi, whereas the sample from the flasks containing fungi was coloured even after only one hour which indicates a very rapid synthesis of IAA. Apart from the shift of the time scale, the curves are otherwise obviously similar. Both reach a maximum fairly rapidly, which is followed by some decrease in IAA concentration. What seems to me to be the most interesting feature is the pronounced second peak—higher than the first—which comes later in both the species, without any further addition of TTP. The phenomenon can hardly be explained by sudden increases of growth rate of the fungi on this sugar-free medium. A possible explanation is that the first IAA peak is reached via one pathway of synthesis and that this is later replaced or supplemented by another more indirect pathway. A secondary activation of the enzymes involved in the synthesis is another possibility.

Finally, an experiment was carried out to determine quantitatively the amount of IAA produced in flasks containing 10.0 mg DL-TTP in the usual NH_4NO_3 medium. *E. myrt.* "Andr." 2 was chosen as the test object, i.e. one of the species which forms IAA most easily. At the same time as these flasks were prepared others were set up with known concentrations of IAA in the same medium, but with no TTP. They contained 0.001, 0.01, 0.1 or 1.0 mg IAA in each flask and were put on a shaker in a dark thermostat with the inoculated flasks. On every measuring occasion the pH in one TTP flask and one flask at each IAA concentration was adjusted to pH 2.9. These were extracted with 50 ml ether in 2 minutes in as identical a way as possible. After adding dry Na_2SO_4, samples of equal size were taken from the ether fractions for chromatography and comparison of the intensity of the spots. This method gives only an approximate idea of the range of concentration of the IAA spot—although it naturally also gives information about what other substances are formed—and has therefore been supplemented by Salkowski measurements. For this, 1 ml samples were taken from the ether fractions. The ether was evaporated off, and then 1 ml distilled water and 2 ml reagent were added. Before the measurements, made after 30 minutes, a further 5 ml water was added, so that the colour intensity would be within a suitable range for measurement. The chromatogram done on a sample taken after nine days showed that the strength of the IAA spot lay between the extracts of 0.1 and 1.0 mg IAA (Plate 2), and this was in good agreement with the colorimetric value which was 82 % of the Z value for 1.0 mg IAA (extracted and treated in exactly the same way as the TTP sample). In this range of concentration the colour intensity was proportional to the amount of IAA added, so that, e. g., 50 % of the Z value for 1 mg IAA was equivalent

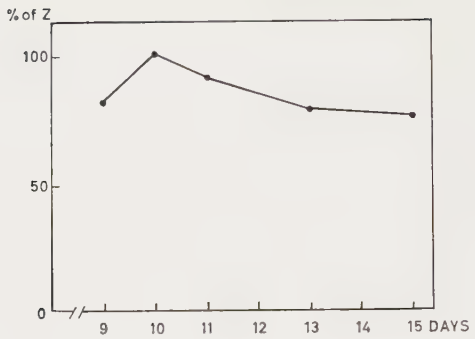


Fig. 24. Quantitative determinations of the production of IAA by *E. myrt.* "Andr." 2 on TTP medium. The spectrophotometrical values with Salkowski reagent are expressed in per cent of the Z value obtained simultaneously for the same volume of a solution containing 1 mg IAA.

to 0.5 mg IAA. Ten days after inoculation, the highest IAA concentration in the medium was reached, as the curve in Fig. 24 shows, viz. 101% of the value for the extract of 1 mg IAA. During the following days the value decreased slowly, to 76% after 15 days. Thus breakdown starts fairly quickly. This has also been shown by earlier curves, but in this fungus probably does not depress the amount of IAA too much.

The maximum value of 101% will now be examined more closely. Since in this case TTP was added to a normal medium containing NH_4NO_3, to ensure good growth—which so small an amount of TTP as 10 mg would not support—a flask with only NH_4NO_3 was extracted in the same way as the other flasks. No colour was obtained with Salkowski reagent, but a weak blue-lilac spot could be detected on the chromatogram when amounts of extract similar to the other samples were applied. However, comparison with extracts of IAA concentrations 0.01 and 0.1 mg showed that the amount of auxin synthesized on NH_4NO_3 was distinctly less than 0.01 mg (Plate 2). Even if it is assumed to be 0.01 mg, there is still exactly 100% of 1 mg IAA which is obtained from TTP.

The Salkowski reaction is not, however, specific for IAA, but a number of other compounds also give a colour. I have therefore tested the colour reactions of some relevant indole compounds and measured the absorption peaks, if they were anywhere near the red region of the spectrum. The result is shown in Table 33.

As can be seen, there are not many of these substances which can affect the result of measurements at 530 mm. The chromatogram (Plate 2) shows that apart from the dominating IAA spot there are some others which can give a colour. The blue spot just after the starting point is probably ILA, as

Table 33. The Salkowski characteristics of some indole compounds and the absorption maximum wavelengths for the resulting colours.

Compound	Colour with reagent	Absorption maximum, nm
IAA	red	525–530
IPya	red (weak)	525–530
IAAld	0	
IAld	brown (weak)	
ICA	pink	480–490
Tryptophol	yellow	
Tryptophan	yellow	
ILA	yellow	
Indole	brown	
IAM	red	530
IAN	blue-lilac	565
IAEt	lilac	530
An. A	0	

was discussed previously, which does not give a red colour with the reagent. Then there is ICA, which colours pink, but the absorption peak does not lie in the same range of wavelengths, so its colour can only increase the measured absorption values by very little, if at all. Then there is IAEt, which is able to interfere. The acid ether fraction has therefore been shaken with 2% $NaHCO_3$, which removes all acid compounds and leaves the neutral ones. However, Salkowski tests on the neutral fraction, done in the same way as before, have given no colour at all. The concentration of IAEt is clearly too low. Consequently only a very small amount of the colour formed can be due to other substances than IAA, so that this can be ignored. The conclusion from this reasoning, therefore, is that *E. myrt.* "Andr." 2 can convert 10% of the TTP added at the beginning. To get a correct figure this value must be multiplied by the ratio of the molecular weights of TTP and IAA, which is 1.17. If the value thus obtained, 11.7, is compared with the result of earlier investigations, it is seen to lie very high. Larsen (1962) writes that the exchange of IAA on TTP medium is usually less than 1% of the theoretical value, in experiments with plant tissue. Brian (1957) also obtained ca. 1% IAA in his experiments with 25 fungi of different genera. Crady and Wolf (1959) found, in their investigations of IAA production by *Taphrina deformans* and *Dibotryon morbosum,* that the former converted 9.5% of the added L-TTP, and the latter 2.4%. The value for *E. myrt.* "Andr." 2 should really be doubled (to 23.4), to make it comparable with these, since it is thought to be only the L form of the amino acid which can serve as a precursor of IAA.

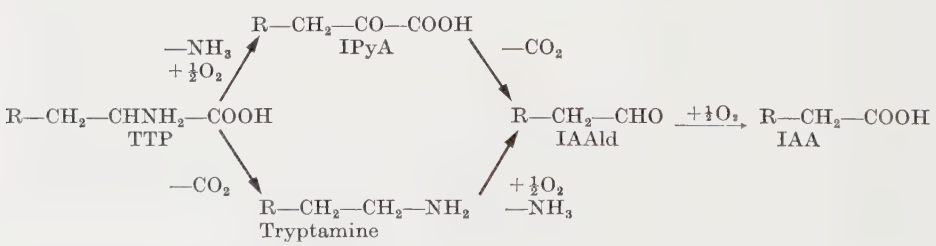
The figures for *Exobasidium* can be compared with the highest (10–30 %), which Libbert *et al.* (1966) could measure in their investigations of the influence of epiphytic bacteria on auxin metabolism. Thin layer chromatograms also here show that IAA is the principal product in the conversion of TTP, and also that ICA can be demonstrated besides at least one unidentified substance. The ability of higher plants to transform TTP to IAA was shown to be very limited.

b. *Results of short term experiments*

The way in which these experiments were carried out has been described in the chapter on methods. The aim of this type of experiment has been to get some indication of which synthetic pathway is used in the different *Exobasidium* species, by adding large amounts of conidia to small quantities of the indole compounds, for short experimental periods.

The preliminary experiments showed, among other things, that sugar can be omitted from the medium, since the fungus has a sufficient nutrient reserve to support necessary metabolism during the period. It was also shown that there was no detectable conversion of TTP, for example, by bacteria or other undesirable microorganisms, even though considerably larger volumes were extracted than those used in the experiments. Therefore, benzylpenicillin sodium, which was added to the flasks at first, was left out in later experiments. Experiments with additions of boiled or unboiled filtrate and with conidia, have given metabolites of TTP only in the flasks with conidia. This shows that it is intra- and not extra-cellular enzymes which are operative. As expected, no spots were obtained on the chromatogram when the conidia had been boiled. This result can be compared with the more surprising one obtained by Konishi and Hagimoto (1961) with sap pressed from the fruit bodies of *Agaricus bisporus*. Although this sap had been boiled, it could convert TTP to IAA, which indicates that it must be a non-enzymatic factor in the sap, which is operative.

According to Gordon and Sanchez-Nieva (1949), the biosynthesis of IAA from TTP is by one of the following two pathways.



In the experiment shown in Plate 3, particular success was achieved in showing, at any rate for *E. myrt.* "Andr." 2, which pathway is followed. It can be seen that the one- and twelve-hour extracts have no spots if no fungus was present, whereas spots from flasks with fungi are clearly seen. Even in the first sample, taken after one hour, there is a brown trailing spot from the starting point to Rf 0.10 and a weak blue-lilac one at Rf 0.30. It was immediately clear that the latter was IAA, but the identity of the former was at first more uncertain. For several reasons, to which I will return later, I am now sure that the substance in question is IPyA, so that the synthetic pathway followed is already indicated. It is even clearer in the 3 hour extract, when it is also possible to observe the next step in the synthesis, i.e. IAAld, which is the brown-lilac spot at Rf 0.46. That this was not already seen in the first sample, in spite of the presence of IAA, is probably because the aldehyde is very rapidly oxidized to the acid, and is therefore always present in very small amounts. In the 6 hour extract no new substances are seen, but to judge from the size of the IPyA spot this reaches its maximum, and it successively decreases in the 9 and 12 hour samples. In the 9 hour sample the next step in the synthesis, i.e. IAAld, reaches its maximum. Only 3 hours later this brown-lilac spot is definitely weaker. It is also clear from the whole series that the IAA spot gets stronger and stronger with each sample, reaching a maximum in the last one. Besides the identification of the compounds involved in the synthesis, the sequence of peak concentrations of these compounds clearly shows that the synthesis of IAA in *E. myrt.* "Andr." 2 is by the uppermost of the pathways shown in the scheme.

Two spots on the chromatograms for extracts from 3 hours onwards have not yet been mentioned. The first one is the yellow spot at Rf 0.40, An. A. It is difficult to know whether this substance takes part in IAA synthesis or is a breakdown product, as has been discussed earlier, but it cannot be seen before the IAA spot is quite visible. The second spot lies just below IAA and is also coloured yellow by Ehrlich's reagent, but the colour always fades away after a few hours. The substance has only been demonstrated in very young cultures.

IPyA has previously appeared difficult to chromatograph without breaking down completely into a number of products. This occurs, for example, in the alkaline solvents which are those most used for the chromatography of auxins. Recently Winter (1964), and others, have used a series of solvents which do not have this effect on IPyA, and which also separate it well from IAA. However, the most suitable of these is not very useful in thin layer chromatography. I have therefore done most of the separations in ethanol (50%) or water, in an atmosphere saturated with acetic acid, and then developed the spot with dinitrophenylhydrazine (DNPH) reagent. Fig. 25

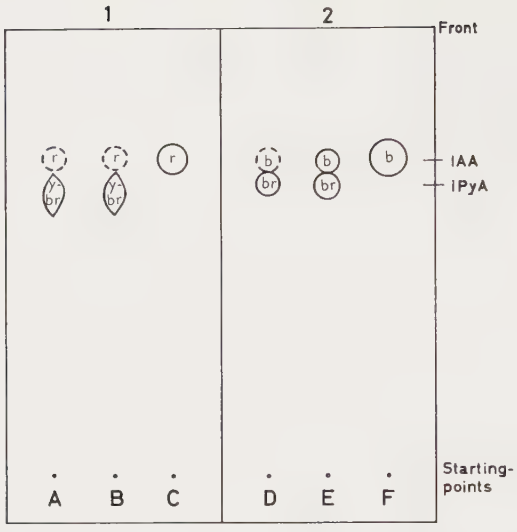


Fig. 25. Chromatograms showing the occurrence of IPyA in the TTP medium, when *E. myrt.* "Andr." 2 had grown in it for 3 (B) and 6 hours (E). Ether extracts (pH 2.9). IPyA (A and D) and IAA (C and F) were run as standards. Solvent ethanol (50 %) with HAc-saturated atmosphere and sprayed with DNPH- (plate 1) and Ehrlich's reagent (plate 2). r = red, y-br = yellow-brown, b = blue and br = brown. A dotted circle means a faint spot.

shows the 3 hour sample from the last experiment (Plate 3), in comparison with IPyA and IAA. The left side shows the result when the plate was sprayed with the reagent. The IAA spot is immediately coloured clear red, whereas the IPyA spot more slowly becomes yellow-brown. The colour and position on the sample agree much more closely with IPyA than with IAA. The 6 hour sample on the right-hand chromatogram in Fig. 25 has been sprayed with Ehrlich's reagent, and is seen to contain more IAA, as mentioned earlier.

After chromatography of extracts from almost all the *Exobasidium* species used in the investigation, grown on TTP, it was clear that the majority of the species which cause only local infections on the host plant synthesize much less IPyA than the systemic forms. This is shown quite well by Fig. 26, for example by a comparison of *E. vacc.* "vit. id." cultures with the systemic ones on the right-hand chromatogram. However, *E. vacc.* "Arct." 3 synthesizes as much IPyA as the closely related but systemic strain, *E. vacc.* "Arct." 4.

IPyA has also shown itself to be very unstable in UV measurements, and after only a few minutes the curve has an appearance different from that previously measured. In Fig. 27, the UV spectrum for the reference substance has been compared with the eluted spot from a chromatogram in water + HAc.

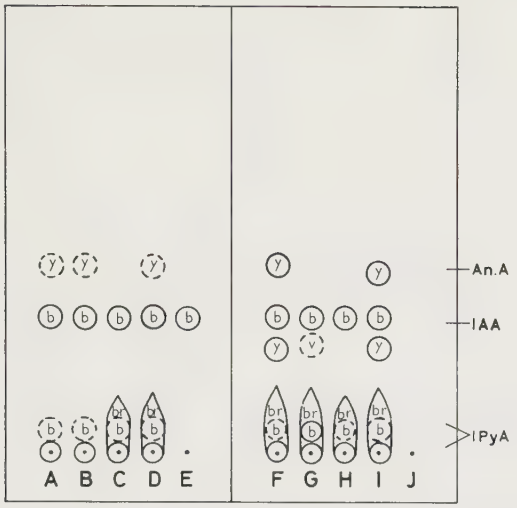


Fig. 26. Various strains of *Exobasidium* were cultivated on a TTP medium for 23 hours, ether extr. at pH 2.9 and chrom. in Solvent S. Ehrlich's reagent. y = yellow, b = blue, br = brown and v = violet. A = *E. vacc.* "vit. id." 6, B = *E. vacc.* "vit. id." 17, C = *E. vacc.* "Arct." 3, D = *E. vacc.* "Arct." 4, E = *E. jap.* 1, F = *E. myrt.* "Andr." 1, G = *E. cass.* 1, H = *E. cass.* 2, I = *E. ang.* 1 and J = *E. myrt.* "ulig." 1.

At first sight the curve from the sample is perhaps not similar to the reference curve, but closer study shows that the characteristic minimum for the substance, at 260 nm, and maximum at 330 nm are also found in the sample. The extra peak at 280 nm is doubtless caused by the conversion to IAA which has taken place in the sample. That this peak can occur is confirmed by measurements of chromatographed synthetic IPyA, shown by the third curve in Fig. 27.

As shown by Fig. 28, IPyA also has a significant biological effect, which is first noticeable at a molarity of 10^{-6} and gives a 49% increase in growth of coleoptiles at 10^{-4} M. A parallel spot to that used in the above-mentioned UV measurement gave, at the same time, 47% increase in growth. According to Bentley *et al.* (1956), the physiological activity of IPyA depends mainly on the products to which it is converted—primarily IAA—but not entirely.

The brown-lilac spots, assumed to be IAAld in Plate 3, also have a clear effect in promoting elongation in the coleoptile test. Even after only 15 minutes incubation with TTP, so much IAAld has been formed by *E. myrt.* "Andr." 2 that a 30% increase in growth can be recorded (Table 34). After 2 hours this figure has risen to 71%, and this can be compared with the 88% increase due to the IAA spot at the same time. That *E. vacc.* "vit. id." 1 can also form considerable amounts of IAAld, in the same time, is shown by the

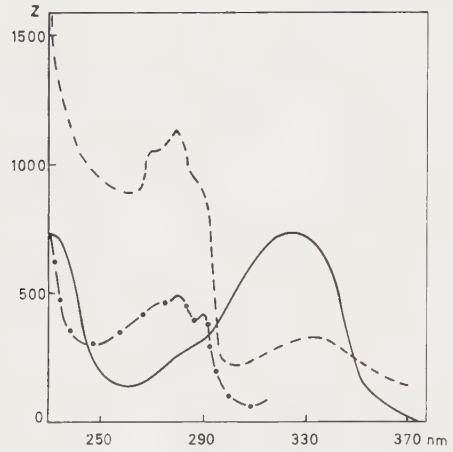


Fig. 27.

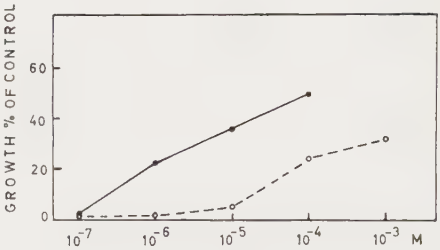


Fig. 28.

Fig. 27. UV absorption spectra of unchrom. IPyA (———), chrom. IPyA (—·—·—·—) and region Rf 0.60–0.75 (coloured yellow-brown with DNPH reagent) of a chromatogram of the acid fraction of an ether extract of *E. myrt.* "Andr." 2 on TTP medium for 23 hours (– – – –). Solvent water, with HAc-saturated atmosphere.

Fig. 28. The response of coleoptiles to various concentrations of IPyA (unbroken line) and tryptophol (broken line).

figure of 53% for increase in growth. The activity of the IAAld depends on its rapid conversion to IAA by the plant tissue.

To bring about an accumulation of metabolites, short term experiments with additions of dimedone have been carried out. This substance blocks IAA synthesis at the step between IAAld and IAA. Different concentrations of dimedone, from 33 to 200 mg per flask, were tested. There was a small increase in IPyA and IAAld, when the fungus was grown on TTP medium. However, it has not been possible to stop IAA synthesis completely, even though high

Table 34. The effect on coleoptiles of parts of chromatograms of ether extracts (pH 2.9) of fungi grown on TTP medium. Solvent S. Ehrlich's reagent.

Fungus	Incubation period, hours	Rf value	Colour with reagent	Growth % of control
E. myrt. "Andr." 2	2	0.28	blue-lilac	+ 88
	1/4	0.45	brown-pink	+ 30
	2	0.45	brown-pink	+ 71
E. vacc. "vit. id." 1	2	0.45	brown-pink	+ 53

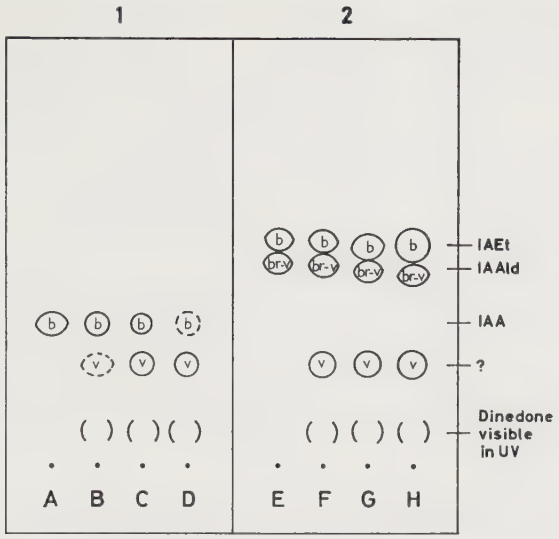


Fig. 29. Chromatograms (Solvent S) showing the effects of different concentrations of dimedone on the auxin production in an 11 days old culture of *E. myrt.* "Andr." 2 on NH_4NO_3 medium. Ehrlich's reagent. b = blue, br-v = brown-violet, v = violet. Acid (plate 1) and neutral (plate 2) ether fraction. The following concentrations of dimedone were used: A and E with 0, B and F with 33, C and G with 100, and D and H with 200 mg per 100 ml culture medium.

concentrations of dimedone were used, indicating that another synthetic pathway is utilized. When dimedone was added instead to sterile experiments with a NH_4NO_3 medium, and the experiment lasted for 11 days, there was a successive decrease in quantity of IAA in the flasks with 33 to 200 mg dimedone per 100 ml medium. In the highest concentration the IAA spot was very weak (Fig. 29). On the chromatogram of the neutral fraction there is, in the sample with no dimedone, a weak brown-lilac spot at Rf 0.42, IAAld, and a clear, blue spot at Rf 0.46, IAEt. With increasing amounts of dimedone, these spots clearly increase in intensity, at the same time as a strong lilac spot, which develops slowly after spraying, makes an appearance at Rf 0.21. The Rf value, the way it develops and is coloured by the reagent, and its lack of auxin effect are evidence that it is IAld. The UV curve of the spot, with maxima at 245 and 278 nm, and a minimum at 260 nm, is similar in some respects to that of IAld (*see* Fig. 15), but the curves cannot be said to be identical, since, among other things, a peak is missing. I cannot therefore say with certainty what the substance is that is accumulated in such a large amount when the normal synthesis is blocked. Another point to be noted is that the amount of IAEt increases with increasing dimedone concentration, at the same time as the quantity of IAA decreases. One can

Table 35. The effect on coleoptiles in TTP medium of addition of conidia from various strains of *Exobasidium*. 6 hours incubation.

Fungus	Growth %	Fungus	Growth %
Control without fungus	38	*E. vacc.* "oxyc." 1	73
E. vacc. "vit. id." 17	58	*E. vacc.* "Arct." 4	71
E. vacc. "vit. id." 2	66	*E. myrt.* "Andr." 2	70
E. vacc. "ulig." 5	67	*E. myrt.* "vit. id." 3	72
E. cass. 2	66		

conclude from this that it is improbable that IAEt is formed from IAA, which is otherwise regarded as normal, but it comes directly from one of the other metabolites.

Another method of blocking the synthesis of IAA from TTP has been used by Klungsöyr (1964). She added $NaHSO_4$, in order to take up the IAAld formed. I have not so far succeeded in completely stopping the formation of IAA using this method. The most obvious result is that very strong An.A spots are obtained on the chromatogram.

Finally, experiments have been done on TTP medium in which coleoptiles were added to dishes containing 0.02 mg TTP in coleoptile nutrient solution and conidia of *Exobasidium* cultures (Table 35). After 6 hours incubation the contents of the dishes and of control dishes with no conidia, were extracted. Chromatograms from control dishes had no spots, but both IPyA and IAA were clearly seen from some of the others, e.g. those containing *E. vacc.* "oxyc." 1 and *E. cass.* 2. The percentage increase in growth can be seen to be distinctly higher in all the dishes, containing fungi, but the differences between the different species is not so large as the chromatograms would lead one to expect. Even those which produce least IAA clearly produce enough to cause increase of growth during the short period of the experiment.

Discussion of TTP metabolism of *Exobasidium,* in which comparisons with other fungal genera will be included, will be deferred until after the metabolism on a number of other indole compounds has been examined.

D. Auxin metabolism on indolepyruvic acid medium

The clearest results with this labile substance were obtained when 1–2 mg IPyA were dissolved in a few drops of ethanol (96%) which was then added directly to the test flasks. The period of incubation with the fungus varied

from 15 to 45 minutes. All the species investigated showed the same sequence of reactions, the one expected, i.e. IPyA → IAAld → IAA. In addition to remaining IPyA, significant amounts of IAA are found in the acid fraction, and the chromatogram of the basic fraction in Solvent I shows a brown-lilac spot at Rf 0.30 and a weak blue one at Rf 0.25. The first has exactly the same position and colour as IAAld chromatographed at the same time, and it is almost equally probable that the blue spot is tryptophol, a substance which is formed on IAAld medium (*see* below). In experiments lasting several hours IAA was eventually obtained even in the control flasks containing no fungi, which shows that IAA synthesis from IPyA readily occurs spontaneously. However, it is clear that *Exobasidium* accelerates the reaction to a high degree.

E. Auxin metabolism on indoleacetaldehyde medium

These experiments also had to be done in the shortest possible time, because in aqueous solution IAAld changes spontaneously, primarily to IAA. However, Larsen and Rajagopal (1964), in their investigations with IAAld, have found that 0.28%, at the most, is converted to IAA in 24 hours at 25°C. In several experiments eight different *Exobasidium* species have been incubated with IAAld for different lengths of time and each time the result has been the same, namely that two clear spots have been obtained on the chromatogram in addition to the brown-lilac IAAld spot, when any was left. The results of all the chromatograms show conclusively that the dark blue spot at Rf 0.28 in S is tryptophol (3-indole ethanol), while the blue-lilac one at 0.32 is IAA. Since the Rf values are so close, there is often only one spot obtained in this solvent, but rechromatography in others shows clearly that there are two different substances. Tryptophol is a very weak auxin at normal concentrations, but at 10^{-3} M it has an auxin effect on coleoptiles as high as 31% (Fig. 28). Larsen (1950 and 1951), who worked with known weights of α-naphtylacetaldehyde and later also with IAAld, found that two molecules of the aldehyde disappeared for every molecule of acid formed. He therefore assumed that a dismutation reaction was involved, in which the substrate served both as a donor and an acceptor of hydrogen ions. Then the reaction with IAAld would take place in the way illustrated below.

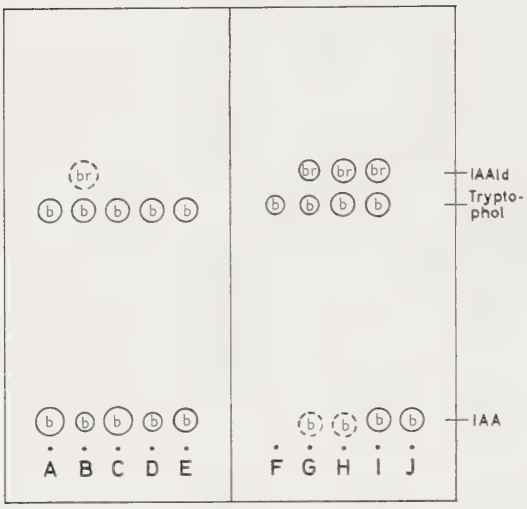


Fig. 30. The rapid conversion of IAAld by three strains of *Exobasidium*. Ether extr. at pH 2.9 and chrom. in petr. ether: chloroform: ethanol (96%) 50:30:20. Ehrlich's reagent. br = brown, b = blue. A = IAA and tryptophol; B = *E. myrt.* "Andr." 2 in IAAld medium for 3/4 hour; C = *E. myrt.* "Andr." 2 in IAAld medium for 5 h; D = *E. vacc.* "vit. id." 17 in IAAld medium for 3/4 h; E = *E. vacc.* "vit. id." 17 in IAAld medium for 5 h; F = Tryptophol; G = *E. vacc.* "ulig." 5 in IAAld medium for 9 min.; H = *E. vacc.* "ulig." 5 in IAAld medium for 14 min.; I = *E. vacc.* "ulig." 5 in IAAld medium for 19 min.; J = IAA.

The substances observed in *Exobasidium* therefore appear to fit into the above sequence of reactions. It is thus probable that IAA and tryptophol are in fact formed in this way. The left-hand chromatogram in Fig. 30 shows clear tryptophol and IAA spots both for *E. myrt.* "Andr." 2 and *E. vacc.* "vit. id." 17, incubated 3/4 and 5 hours with IAAld. However, the right-hand chromatogram shows that when as short experimental periods as 9–19 minutes were used both tryptophol and IAA were formed, but the tryptophol spots are much clearer than the IAA spots. I think that such a difference in intensity of colour indicates that tryptophol is formed before IAA and not at the same time, as the scheme above implies. The intensity with Ehrlich's reagent is about the same for both compounds.

When IPyA metabolism was discussed, it was suggested that tryptophol was also found there in small amounts, but on TTP medium it has not been possible clearly to demonstrate this compound, which seems strange, if IAA metabolism is via IPyA and IAAld. Libbert and Brunn (1961), in their studies with an enzyme obtained from *Pisum sativum*, which could convert TTP to IAA, encountered a substance not at first identified, but later identified as tryptophol. However, this was first possible when aldehyde oxidase,

which normally converts IAAld to IAA, was blocked with a native inhibitor. When the inhibitor was removed the increased amount of tryptophol was oxidized to IAA via IAAld. In *Exobasidium,* added tryptophol is also converted to IAA, as will be seen later, but the reaction is not especially rapid.

F. Auxin metabolism on tryptamine medium

Investigations using tryptamine as a nitrogen source have been carried out for two reasons. The first is that tryptamine has been assumed to be an alternative pathway in IAA synthesis from TTP (as shown in the scheme on p. 62). The second is that, even if tryptamine is not such an intermediate, it has been found in plants (Stowe, 1959, and others) and can therefore serve as a substrate for the synthesis of other indole compounds.

The assumption that tryptamine is a metabolite in IAA synthesis, between TTP and IAAld, may be discussed first, because in spite of careful investigation of the basic fraction, where evidently at least a part of the tryptamine remains, I have found no trace of it. Perley and Stowe (1966) have recently studied the ability of different microorganisms to make use of this synthetic pathway. According to their paper, there are only two relevant reports of microorganisms (however, *see* Hirata (1958) below) which can form tryptamine when they are grown on a medium containing TTP. One of these, concerned with fungi, is Crady and Wolf's (1959) previously cited work with *Taphrina deformans* and *Dibotryon morbosum.* Perley and Stowe, even with the same fungal cultures, showed that no substance resembling tryptamine could be found, even though they used considerably more careful methods. However, IPyA was found, and the authors therefore conclude that the formation of IAA goes via this substance. The other report of tryptamine formation is for bacteria, and here there is no doubt that the substance is produced. According to A. Winter (personal communication, 1966) it is also very doubtful, to judge from his research, whether the synthetic pathway via tryptamine is operative in higher plants. Results which previously seemed to show that it was, are probably due to the presence of bacteria in the tissues investigated. However, it can also be mentioned that it seemed to Hirata (1958) that *Protomyces* may produce tryptamine and IAAld as the intermediates in the metabolic process of TTP, because some similar spots to the specific intermediates in respect of colour reaction and Rf value were shown in the paper chromatogram.

The following result has been obtained with different *Exobasidium* species in short and long term experiments on tryptamine medium. It was already clear that *E. myrt.* "Andr." 1 and 2 were in a special position, in that tests

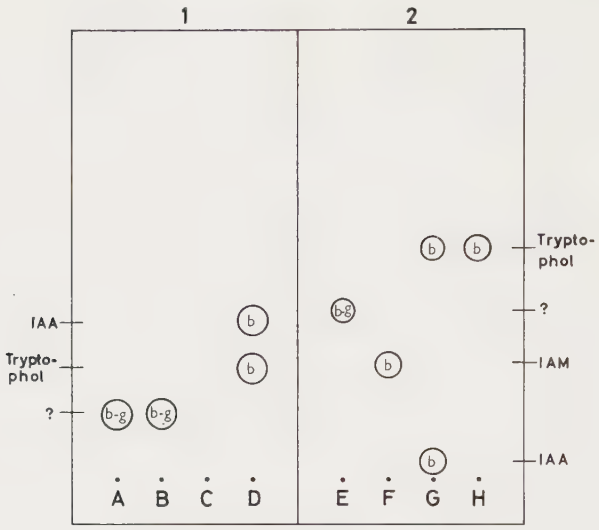


Fig. 31. Some strains of *Exobasidium* were cultivated on tryptamine medium for 19 hours' ether extr. at pH 2.9 and chrom. in Solvent S (plate 1) and in petr. ether: chloroform: ethanol (96 %) 50:30:20 (plate 2). Ehrlich's reagent. b = blue, b-g = blue-green. A = *E. vacc.* "ulig." 5, B and E = *E. vacc.* "vit. id." 12, C = *E. Karst.* 3, D and G = *E. myrt.* "Andr." 2, F = IAM and H = tryptophol.

with Salkowski reagent gave a very clear positive reaction with these cultures, but not with the other species tested. The conclusion was drawn that this was the only species which could utilize this nitrogen source for IAA production. Later investigations with other species, and chromatography of the extract, have, however, led to a partial modification of this idea.

Short term experiments, i.e. lasting less than 24 hours, have all clearly shown that there are two alternative pathways for the synthesis of indole compounds from tryptamine. One is represented in *E. myrt.* "Andr." 1 and 2, which, in a short period, form tryptophol and IAA, i.e. the same compounds which could be formed by all *Exobasidium* species on IAAld medium. In Solvent S, these substances here have Rf values of 0.26 and 0.35, respectively, as shown in Fig. 31. The left-hand chromatogram in this photograph also shows the blue spot which demonstrates the other synthetic pathway. This spot, which usually has Rf 0.18, but which lies unusually low on this chromatogram, is formed by all other *Exobasidium* species tested, and in most of them it is the only detectable metabolite. Although this spot has been compared with all available reference substances, it is still not clear what substance it is. Like tryptophol, it belongs to the neutral fraction, but partly goes over in the ether in extractions at pH 2.9. It is coloured more blue-

Table 36. The effect on coleoptiles of parts of a chromatogram of ether extract (pH 2.9) of *E. myrt.* "Andr." 2 on tryptamine medium (24 hours). Solvent S. Ehrlich's reagent.

Rf values	Colour with reagent	Growth % of control
0.16–0.21	blue	+ 3
0.21–0.26	0	+ 2
0.26–0.31	blue-lilac	+57
0.46–0.51	0	+ 2

green than tryptophol, which is pure blue, and in most solvents it moves so close to tryptophol that confusion may easily arise. However, the right-hand chromatogram in Fig. 31 shows clearly that two separate substances are involved. Neither is the unknown substance the same that was accumulated in the above-mentioned experiments with dimedone.

The coleoptile tests also demonstrate clearly that IAA is formed from tryptamine. The extract used in Table 36 is from a 24 hours culture of *E. myrt.* "Andr." 2 on tryptamine and the result shows that the time was sufficient for enough IAA to be formed to increase growth considerably. In contrast, the tryptophol spot (Rf 0.16–0.21) has no effect. That this is in fact tryptophol is shown not only by all the chromatography data but also by the UV spectrum in Fig. 32.

Although only *E. myrt.* "Andr." cultures have appeared able to form IAA in short experiments, a surprising result was obtained in another type of experiment. This was done with coleoptiles in tryptamine medium to which was added washed conidia of three fungi. The tryptamine was dissolved in

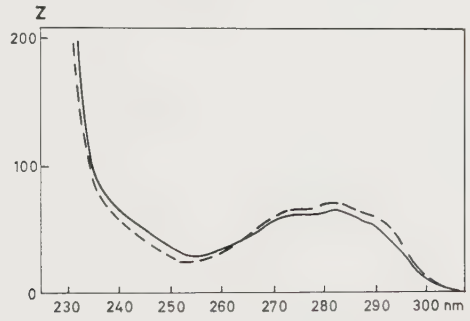


Fig. 32. UV absorption spectra of chrom. tryptophol (unbroken line) and zone 0.18–0.25 (coloured blue with Ehrlich's reagent) of a chromatogram with the basic (pH 8.1) ether extract of *E. myrt.* "Andr." 2 on tryptamine medium for 3 days (broken line). Solvent I.

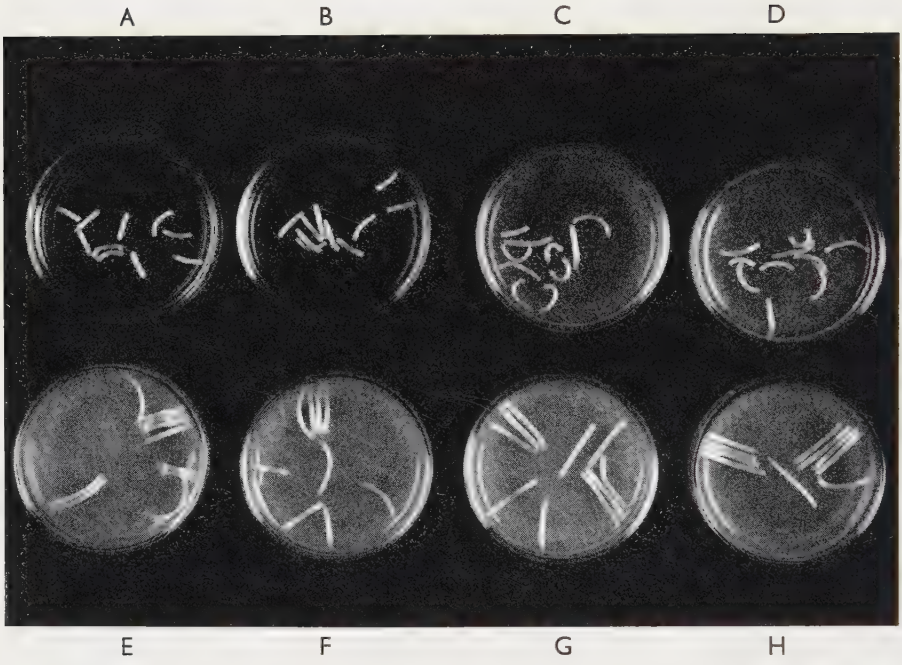


Photo. 1. *See* table 37. Dishes A–B = controls without fungus, C–D = *E. myrt.* "Andr." 2, E–F = *E. vacc.* "vit. id." 17, G–H = *E. vacc.* "ulig." 5. Ca. 1/2 x.

the smallest possible volume of ethanol but even so this meant that the coleoptiles in the control dishes grew badly, but those in the fungi-containing dishes did well. For this reason I have not calculated the percentage growth values. The result of the experiment is shown in Photo 1, as well as in Table 37. The coleoptiles in the dishes with *E. myrt.* "Andr." 2 grew less well than was expected, but this is certainly because the auxin concentration was much too high, as indicated by the twisted appearance of the coleoptiles. However,

Table 37. The effect on coleoptiles in tryptamine medium of addition of conidia from various strains of *Exobasidium.*

Fungus	Average growth, mm
Control without fungus	2.7
E. myrt. "Andr." 2	5.1
E. vacc. "vit. id." 17	9.1
E. vacc. "ulig." 5	10.7

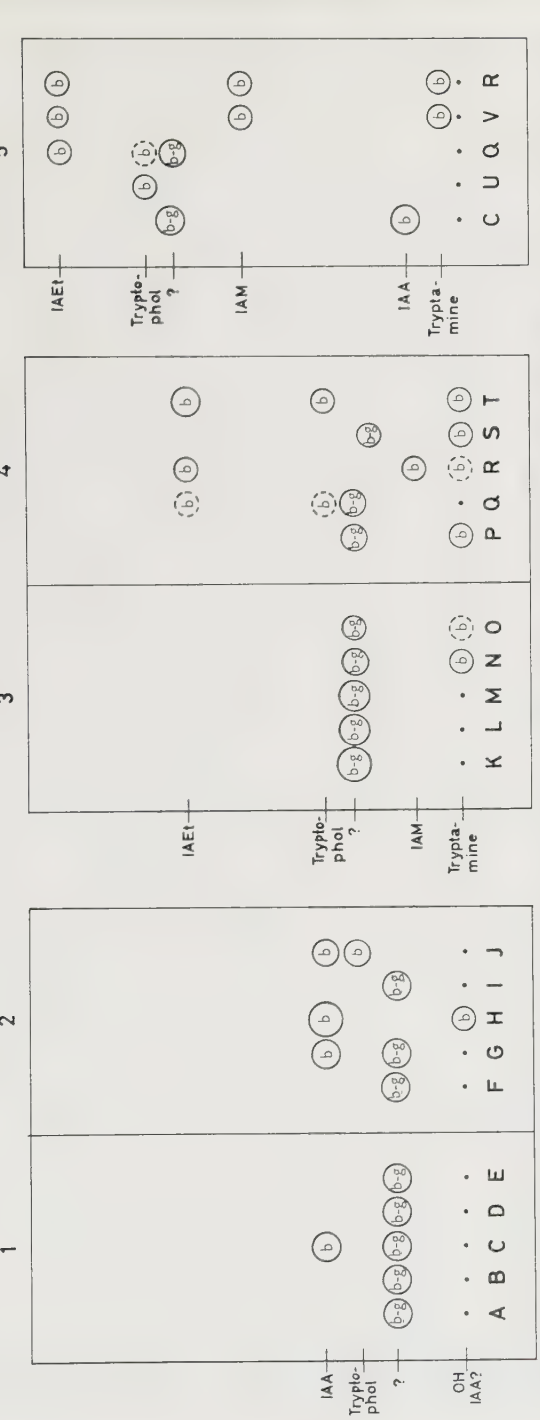


Fig. 33. The auxin metabolism on tryptamine medium. 9 days old cultures were extracted with ether at pH 2.9 (A–I) and 8.1 (K–S) and chrom. in Solvent S (plates 1 and 2), Solvent I (plates 3 and 4) and Solvent V (plate 5). Ehrlich's reagent. b = blue, b-g = blue-green. A and K = *E. vacc.* "vit. id." 2, B and L = *E. vacc.* "vit. id." 17, C and M = *E. vacc.* "ulig." 5, D and N = *E. uvae ursi* 2, E and O = *E. cass.* 2, F and P = *E. myrt.* "myrt." 2, G and Q = *E. myrt.* "vit. id." 3, H and R = *E. myrt.* "Audr." 2, I and S = *E. vacc.* "Arct." 4, J = tryptophol and IAA, T = tryptamine, tryptophol and IAEt, U = tryptophol and V = tryptamine, IAM and IAEt.

the surprising thing is the clear growth stimulation caused by *E. vacc.* "vit. id." 17 and *E. vacc.* "ulig". 5. The figures are at the same level as those usually reached by the very strongest auxins. The experiment was also followed up by extraction of the media in the dishes. Although three solvents were used, the chromatograms showed only the spots which would have been there even in the absence of the coleoptiles, i.e. tryptophol and IAA in *E. myrt.* "Andr." 2 and the blue-green spot in the other two. Possible explanations of the large amount of growth might be that small but sufficient quantities of IAA were formed at the same time (*see* below), or that the coleoptiles can convert the unidentified substance to IAA.

What happens when *Exobasidium* is incubated with tryptamine for a longer experimental period can most easily be described by considering the experiment in Fig. 33. The extract taken after three days, which is not shown in the photograph, gave largely the same result as the short term experiments. After 9 days, part of the content of the flasks was tested with Salkowski reagent. A clear red colour was formed in *E. myrt.* "Andr." 2, but a weaker, pink colour was obtained with *E. myrt.* "vit. id." 3 and, surprisingly, with *E. vacc.* "ulig." 5. The chromatogram of the acid fractions confirms that IAA is also formed in clear amounts in *E. myrt.* "vit. id." 3 and *E. vacc.* "ulig." 5, even though they form the unidentified substance, as do the other species tested, except for *E. myrt.* "Andr." 2. The spots of this are seen even better in the basic extract. In addition to this substance, *E. myrt.* "vit. id." 3 forms small amounts of tryptophol, and is the only species found so far which can form both these compounds. The fact that they are both there shows even more clearly that there really are two different substances. In *E. myrt.* "Andr." 2, besides the small amount of remaining tryptamine at the starting point, there is a blue spot at Rf 0.12 and another blue one at 0.61. There is no doubt that these are indoleacetamide (IAM) and IAEt respectively, as shown by reference substances chromatographed at the same time on the same and the following chromatogram. The tryptophol spot, previously so clear, is now absent, which shows that this early stage in the reaction has been passed. IAM and IAEt are very probably products of the conversion of IAA. As shown by Fig. 33, IAEt is also formed in small quantities in *E. myrt.* "vit. id." 3.

Thus, in summary of the tryptamine studies it can be stated that most *Exobasidium* species form a substance as yet unidentified, which except in *E. vacc.* "ulig." 5, has not appeared to be able to lead to IAA. It is less certain whether it does so in *E. myrt.* "vit. id." 3, since there tryptophol is formed as well as the unidentified substance. In *E. myrt.* "Andr." cultures (and possibly other *E. myrtilli* forms belong here), tryptophol and IAA are formed in a way that indicates that it is via IAAld. However, IAAld has

not clearly been demonstrated. Sastry and Muir (1965) have also concluded that tryptamine must undergo conversion to IAA by a pathway which does not include an aldehyde as intermediate.

G. Auxin metabolism on indolelactic acid medium

As mentioned earlier, small amounts of a substance which was probably ILA were encountered when the fungi were grown on TTP medium. As early as 1911, Ehrlich and Jacobsen showed that this substance could be formed by fungi from TTP through deamination. Later, Kaper and Veldstra (1958) showed ILA in *Agrobacterium tumefaciens* and Perley and Stowe (1966) have shown that *Taphrina deformans,* and Bailey and Gentile (1962) that *Diplodia natalensis* can also form this substance. In *Diplodia,* ILA is even the dominating final product on TTP medium, and IAA is only formed in small amounts. The same thing has later been reported for *Endomycopsis vernalis* by Glombitza and Hartmann (1966), who considered both ILA and tryptophol to be final products.

To see if ILA is a probable metabolite in *Exobasidium,* both long and short term experiments have been done. In general, it can be said that metabolism on ILA is very sluggish compared with that for most other indole compounds. Experiments of less than 24 hours give no clear spots at all on the chromatogram, but then weak IAA spots gradually appear, in most of the species investigated. The synthesis was most rapid in *E. myrt.* "Andr." 2 and *E. vacc.* "vit. id." 6. That it really is IAA which is formed is shown, for example, by UV spectrophotometric measurements. Long term experiments (up to 16 days) have yielded IAA as the only detectable metabolite in all the fungi except *E. myrt.* "Andr." 1 and 2, but never in any large quantity. In these two cultures IAEt is also formed. Among the substances absent from the chromatogram in all my experiments, the decarboxylation product of ILA, i.e. tryptophol, should be mentioned first, for this substance has also been detected in those cases when ILA was found in TTP media. Wightman (1964) found considerable amounts of tryptophol when he studied ILA metabolism of tomato. He therefore assumed that ILA was decarboxylated to tryptophol which was then oxidized to IAA. Since tryptophol would certainly have been detected if it had been formed in *Exobasidium,* I do not think it probable that the reaction occurs in this way in this organism.

H. Auxin metabolism on tryptophol medium

Although tryptophol was not detected when *Exobasidium* was grown on ILA medium, it was shown, as previously mentioned, in tryptamine, IPyA and IAAld media.

Table 38. The effect on coleoptiles of parts of chromatograms of ether extracts (pH 2.9) of fungi incubated 18 days on tryptophol medium. Solvent S. Ehrlich's reagent.

Fungus	Rf values	Colour with reagent	Growth % of control
E. myrt. "Andr." 2	0.48–0.58	blue	+90
E. vacc. "vit. id." 1	0.48–0.58	blue (weak)	+64
E. vacc. "vit. id." 6	0.48–0.58	blue (weak)	+62

Short term experiments showed that of the ten species tested on this substrate all except two formed IAA quite easily. That *E. ang.* 1 and *E. vacc.* "Arct." 3 did not do so might be because they were not in as good condition as the others at the time of the experiment. However, there was no evidence showing that they were not. In longer experiments considerable amounts of IAA were formed. Most was formed by *E. myrt.* "Andr." 2, in which the tryptophol was used up after a time. In all the others there was still a lot of tryptophol left when the experiment finished after 18 days. Only one other substance was clearly formed, besides IAA, viz. IAEt. As expected, the largest amounts were for *E. myrt.* "Andr." 2, but although the spots were weak there is no doubt that *E. vacc.* "vit. id." 1 and 6 also form this substance. The coleoptile test gives a clear positive result for the relevant zone of the chromatogram (Table 38).

I. Auxin metabolism on indolepropionic acid and indolebutyric acid media

The work of Fawcett *et al.* (1958) shows that IAA can also be formed by β-oxidation of indole acids with long side chains. Stowe (1959) has not yet demonstrated this with certainty for higher plants, but provisional identifications of IPrA and IBA have been made. It has also been shown that IPrA can be formed from TTP by bacteria. Some of the synthetic acids with an even number of carbon atoms in the side chain, e.g. IBA, have been broken down to IAA, but those with an uneven number of carbon atoms could only be shortened to IPrA.

In my investigations of the ability of *Exobasidium* to perform such a breakdown, three species have so far been grown on such media. All three have given the same result. After 22 hours incubation the chromatogram has the appearance shown in Fig. 34. In IBA medium (the last spots) a blue spot

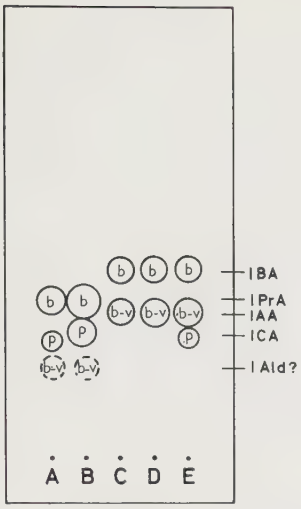


Fig. 34.

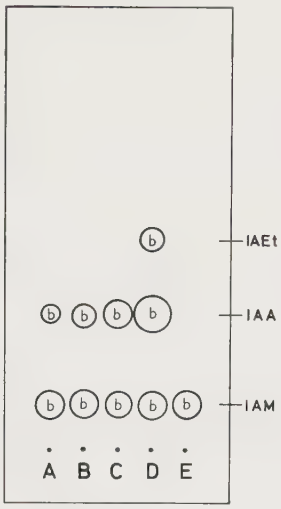


Fig. 35.

Fig. 34. The decomposition of IPrA and IBA after 22 hours by *Exobasidium*. Ether extr. at pH 2.9 and chrom. in Solvent S. Ehrlich's reagent. b = blue, b-v = blue-violet, p = pink. A = *E. vacc.* "vit. id." 3 in IPrA medium; B = *E. vacc.* "vit. id." 1 in IPrA medium; C = *E.vacc.* "vit. id." 3 in IBA medium; D = *E. vacc.* "vit. id." 1 in IBA medium; E = ICA, IAA and IBA.

Fig. 35. The conversion of IAM by *Exobasidium*. Ether extr. at pH 2.9 and chrom. in Solvent S. Ehrlich's reagent. b = blue. A = *E. vacc.* "vit. id." 3 incubated 7 hours in IAM medium; B = *E. vacc.* "vit. id." 3 incubated 23 hours in IAM medium; C = *E. myrt.* "Andr." 2 incubated 7 hours in IAM medium; D = *E. myrt.* "Andr." 2 incubated 23 hours in IAM medium; E = Control without fungus. 23 hours.

at Rf 0.30 was obtained and this can hardly be anything other than IAA, which thus is in agreement with the earlier investigations. As expected, IAA was not formed from IPrA, but instead there was a yellow-pink spot at Rf 0.28. Since the Rf value and the colour agree fully with those of ICA, there seem to be grounds for assuming that *Exobasidium* can perform the β-oxidation as far as it is possible, i.e. down to ICA. Under the pink spot there is always a very small blue-lilac spot, which may be IAld.

J. Auxin metabolism on indoleacetonitrile medium

As mentioned earlier, I previously thought that the very active auxin encountered time and time again in the neutral fraction was IAN. However, rechromatography of these extracts has shown that it is almost always IAEt. Yet in one experiment on TTP medium a clear substance was obtained which

agrees fully with IAN. This could not be repeated, so this substance has not been included among those mentioned as encountered on TTP medium. In any case, incubation experiments with IAN were carried out with *Exobasidium,* since IAN was thought to be a possible precursor of IAA. It has also been found in several higher plants, especially in various cabbage species, where it is readily formed through breakdown of the glycoside glucobrassicin. Libbert and Ballin (1959) have shown that IAN affects cell elongation in two ways. Either IAN is hydrolysed to IAA, as takes place in *Triticum,* thereby explaining the elongation in wheat leaves and coleoptiles, or else it has itself an effect (e.g., in *Impatiens*). I draw the following conclusions from my own experiments. All the short term experiments, and one 10-day experiment, have, after extraction, shown only large IAN spots on the chromatogram. In the longest sterile experiments a very weak IAA spot was obtained only after 13 days in *E. myrt.* "Andr." 2, and after 16 days in *E. vacc.* "vit. id." 17 and possibly also in *E. myrt.* "myrt." 2. In the first two, ICA was also present, and rechromatography in III showed that the amount of ICA was considerably greater than of IAA, which then appeared very weak. This agrees well with what Ballin (1962) found for higher plants, in which IAN, like other nitriles, is broken down, through α-oxidation with loss of a C atom, to IAld and ICA. Whether there was any trace of IAM, the expected intermediary product in a hydrolysis of IAN, was investigated with especial care.

$$\underset{\text{IAN}}{R{-}CH_2{-}CN} \xrightarrow{H_2O} (\underset{\text{IAM}}{R{-}CH_2{-}CO{-}NH_2}) \xrightarrow{H_2O} \underset{\text{IAA}}{R{-}CH_2{-}COOH} + NH_3$$

Like Thimann and Mahadevan (1958), I could not detect this metabolite in the medium.

K. Auxin metabolism on indoleacetamide medium

To find out, among other things, whether the second step in the above reaction goes as slowly as the first, experiments with IAM as the nitrogen source were carried out. As shown by the chromatogram in Fig. 35, both *E. vacc.* "vit. id." 3 and *E. myrt.* "Andr." 2 converted IAM to IAA in a short time. Very clear IAA spots were already visible in the 7 hour extract, and in the 23 hour extract the IAA spot is larger than that for IAM in *E. myrt.* "Andr." 2. In this species IAEt is already present at this stage. What happens after continued incubation is shown by a long term experiment including other fungi. IAM is rapidly used up by *E. myrt.* "Andr." 2 and *E. vacc.* "vit. id." 17, but some remains during the whole experimental period in

E. myrt. "myrt." 2, which, as in all previous experiments, had a very slow auxin metabolism, only partly explained by the fact that growth at the beginning is always slow. Besides in *E. myrt.* "Andr." 2, in all samples, IAEt also occurs weakly in the 6 days sample of *E. vacc.* "vit. id." 17, but is absent from the later extracts. The time of the appearance of this substance, and the experiments with IAA (*see* below) indicate that the ester is a product of the conversion of IAA.

Even if the synthetic pathway via IAN to IAM is not thought to operate, the role of IAM has again become topical through the publication of works by Riddle and Mazelis (1965), who have succeeded in showing a peroxidase-catalysed decarboxylation of TTP to IAM in preparations of cabbage, and by Magie *et al.* (1963), who have demonstrated a conversion of L-TTP into IAA including only IAM as an intermediate in *Pseudomonas savastanoi.*

L. Auxin metabolism on indoleacetic acid medium (breakdown of IAA)

It has long been known that IAA can be broken down photolytically by the action, for example, of riboflavin (B_2). According to Meyer (1956) this occurs in the following way:

IAA → IAld → ICA → An.A → glycol → oxalic acid → formic acid

As early as 1934, Thimann assumed that IAA could be broken down by other means than the effect of light, and he demonstrated this by adding leaf extract of *Vicia faba.* Since then there have been numerous publications about attempts to break down IAA by enzymatic processes. Up to the ICA step, this breakdown takes place according to the above scheme, but in 1961 (Müller) it was still not clear whether the breakdown involved the opening up of the indole ring, via An. A.

It seemed to me important to investigate the reactions of the different *Exobasidium* species to IAA, because any oxidases present will affect not only the amount of IAA produced by the fungus itself, but also the level of endogenous IAA in the host plants. The short term experiments led to the conclusion that none of the *Exobasidium* species can rapidly convert IAA. The only substance which can be identified with certainty is IAEt in *E. myrt.* "Andr." 2. Two sterile long term experiments were carried out, and in connection with the extractions from the flasks progressive quantitative determinations of the IAA content were made by the Salkowski method. Values for *E. myrt.* "Andr." 2, *E. myrt.* "vit. id." 3, and *E. vacc.* "vit. id." 2 are included in Table 39. A decrease of the amount of IAA (at 530 nm) was

Table 39. The decomposition of IAA by various strains of *Exobasidium*. Salkowski reagent was added and the intensity of the colour measured.

Wavelength, nm	Incubation period, days	Z-values for *E. myrt.* "Andr." 2	*E. myrt.* "vit. id." 3	*E. vacc.* "vit. id." 2
530	5	500	520	500
500	7	410	425	390
510	7	455	470	430
520	7	490	510	465
525	7	500	520	470
530	7	495	520	470
500	12	270	260	135
510	12	280	275	140
520	12	285	290	142
525	12	285	290	140
530	12	285	290	135

first detectable 7 days after inoculation, and then only to a slight extent, in *E. vacc.* "vit. id." 2. However, after 12 days, significant breakdown had started. Then the IAA contents of flasks with cultures of *E. myrtilli* are about the same, whereas those of *E. vacc.* "vit. id." 2 are considerably lower. In this species the absorption maximum had also been shifted somewhat downward, to lie at 520 nm, and this is probably because the amount of ICA formed here was the greatest and affected the appearance of the curve. The absorption curve of ICA has a peak at about 490 nm (*see* Table 33). That the amount of ICA was greatest in *E. vacc.* "vit. id." 2 was confirmed by the chromatogram, but the other species also formed small quantities (Fig. 36). The other chromatogram in this Fig. shows most of the other products obtained on IAA medium. In *E. vacc.* "vit. id." 2, *E. vacc.* "oxyc." 1 and *E. myrt.* "vit. id." 3 there are weak spots at about Rf 0.70. These are IAld, i.e. the metabolite between IAA and ICA. They are coloured yellow by van Eck's reagent, which is specific for IAld. *E. myrt.* "Andr." 2 does not have this spot, but has two clearer ones, IAEt and IAM. The amide is formed when there is a good supply of IAA. The chromatograms in the second long term experiment showed the same products, in the main, but in *E. myrt.* "Andr." 2 there was another spot, which in three different solvents (S, Sp and A) gave the same Rf value as OH-IAA. This substance has been identified but not isolated from plants. The presence of the hydroxyl group means that the compound is not a significant auxin.

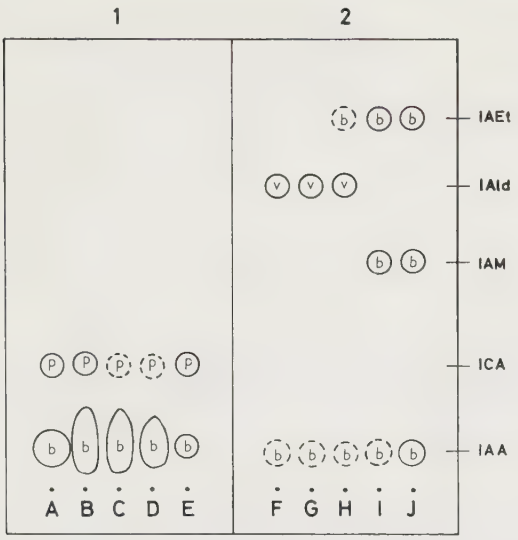


Fig. 36. The indole compounds found in 12 days old cultures of four *Exobasidium* strains incubated in IAA medium. Ether extr. at pH 2.9 (plate 1) and 8.1 (plate 2) and chrom. in Solvent V. Ehrlich's reagent. b = blue, p = pink, v = violet. A and F = *E. vacc.* "vit. id." 2, B and G = *E. vacc.* "oxyc." 1, C and H = *E. myrt.* "vit. id." 3, D and I = *E. myrt.* "Andr." 2, E = IAA and ICA and J = IAA, IAM and IAEt.

In forming IAEt *E. myrt.* "Andr." 1 and 2 are in a class of their own and produce the substance on almost all media. In the experiments with IAA, as on other occasions when there is a large amount of IAA formed, e.g. on IAAld, IAM and N-Z case media, weak spots of IAEt are also seen from time to time in other *Exobasidium* species. Coleoptile experiments like that shown in Table 40 show that there can be a clear auxin effect even though no spots are visible on the chromatogram. The IAEt zone has been

Table 40. The effect on coleoptiles of parts of chromatograms of ether extracts (pH 8.1) of *E. myrt.* "Andr." 2 on IAA medium for 21 hours (A); *E. vacc.* "vit. id." 3 on NH_4NO_3 medium without (B) and with (C) additions of IAA for 24 hours; and IAEt (D). Solvent S. Ehrlich's reagent.

Adding	Rf values	Colour with reagent	Growth % of control
A	0.45–0.60	blue	+ 131
B	0.45–0.60	0	− 1
C	0.45–0.60	0	+ 54
D	0.45–0.60	blue	+ 94

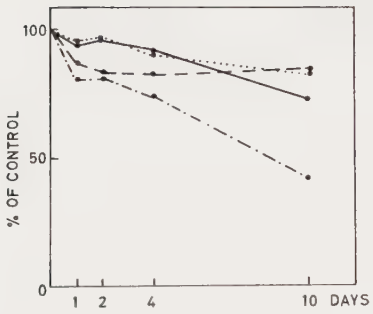


Fig. 37. The decomposition of IAA by *E. uvae ursi* 2 (– – –), *E. vacc.* "oxyc." 1 (·····), *E. myrt.* "Andr." 2 (———) and *E. vacc.* "ulig." 5 (—·—·—). Measured spectrophotometrically after the addition of Salkowski reagent. The Z values expressed in per cent of those of controls with initial amounts of IAA.

scraped from the chromatograms of extracts from flasks containing NH_4NO_3 media with and without the addition of 1 ml 10^{-5} M IAA solution, in which *E. vacc.* "vit. id." 3 had grown. As usual, flasks with only NH_4NO_3 caused no increase of coleoptile growth, but the result for the IAA flasks was +54%. The experiment also shows the effect caused by *E. myrt.* "Andr." 2 after it has been incubated on IAA-medium, and that IAEt really retains its auxin effect even when it has been chromatographed.

The breakdown of IAA has been studied most carefully in an experiment (Fig. 37), in which the same technique was used as in the quantitative determination of IAA production on TTP medium (Fig. 24). After 10 days the level of IAA was still high in *E. uvae ursi* 2, *E. vacc.* "oxyc." 1, and *E. myrt.* "Andr." 2, whereas there was considerably more breakdown in *E. vacc.* "ulig." 5, reminding one of *E. vacc.* "vit. id." 2 in Table 39. The values for *E. vacc.* "oxyc." 1 are surprisingly high, perhaps here also because it grew less well than the other species. However, it is fairly clear that my assumption that there would be more breakdown in most of the *E. vaccinii* cultures, based mainly on the clearer ICA spots in these, is verified by these two experiments. The relationships are somewhat similar to that found by Ulrich (1960), in her investigations of mycorrhiza-forming fungi, for a group of fungi represented by *Coprinus comatus*, which has a very effective IAA oxidase system.

M. Auxin metabolism on indolealdehyde medium

Exobasidium was also incubated with IAld, to enable further study of the breakdown products of IAA to be made. Firstly, the experiments showed

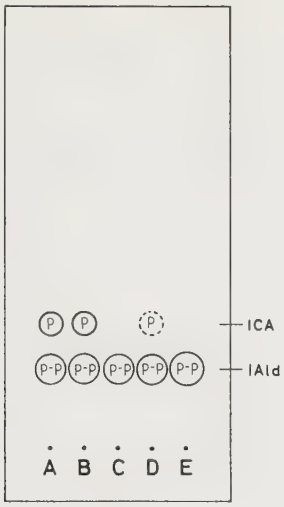


Fig. 38. Four strains of *Exobasidium* grown in IAld medium for 17 hours, ether extr. at pH 2.9 and chrom. in Solvent S. Ehrlich's reagent. p = pink, p-p = pale pink. A = *E. vacc.* "ulig." 5, B = *E. vacc.* "vit. id." 12, C = *E. Karst.* 3, D = *E. myrt.* "Andr." 2 and E = control without fungus.

that IAld is only a breakdown product, when it is present in the extract, e.g. on TTP medium, and cannot serve as a substrate for IAA formation, as no traces of IAA were detected. However, oxidation of IAld to ICA is fairly rapid, and after only four hours I have seen clear traces of the acid. There are distinct differences between the species, shown by the chromatogram in Fig. 38. The experiment from which the chromatogram was made was done with two species which form localized spots on the host, *E. vacc.* "ulig." 5 and *E. vacc.* "vit. id." 12, and two systemic species, *E. Karst.* 3 and *E. myrt.* "Andr." 2. After incubation for 17 hours, very clear ICA spots were seen for the first two species, but they were almost completely absent from the systemic forms.

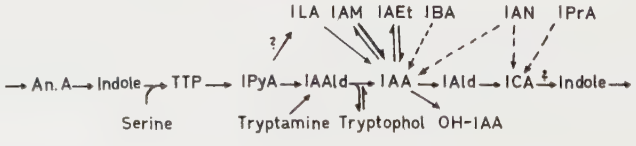
There were also other differences. In long term experiments there were blue-lilac spots for *E. vacc.* "vit. id." 17, *E. vacc.* "ulig." 5 and *E. vacc.* "Arct." 4 but not for *E. myrt.* "Andr." 2. The substance has been compared with indole in three solvents and agrees well in all of them. This indicates that these species, at any rate, can carry out the last carboxylation. However, there were no traces of An.A, which, as earlier mentioned, has been shown in photolysis experiments, and which is usually easy to detect even in small amounts. This was also the case for my incubation experiments with indole.

N. Auxin metabolism on IAA ethyl ester medium

The last incubation experiments were with IAEt. According to Stowe (1959), this auxin probably occurs in higher plants in which an enzyme which can form the ester from IAA has been found. Short term experiments have shown that IAA is formed rapidly in IAEt medium in the presence of any of the investigated *Exobasidium* strains, but long term experiments were necessary to see subsequent development. This can be seen from 2nd to 16th day in Fig. 39. Even after two days there are visible differences in rate of conversion by the tested species and after six days this tendency is still clearer. The quantities of IAA formed by *E. vacc.* "vit. id." 17 are considerably higher than for both the others. In this species the IAEt gradually disappears completely, as it does also in the slower *E. myrt.* "myrt." 2, but there is always some left in *E. myrt.* "Andr." 2, possibly because this fungus forms more IAEt from the large amount of IAA which is now present in the flasks. The 13 and 16 day samples also show that the breakdown proceeds further in *E. vacc.* "vit. id." 17 than in the others, so that clear ICA spots are seen on chromatograms 4 and 5. A blue-green spot is also easily seen, but it has not been possible to identify it. One possibility is that it is indoleglycolic acid (R-CHOH-COOH), which is assumed to be a metabolite, not previously discussed, between IAA and IAld (Glombitza and Hartmann, 1966). It has not been possible to obtain the reference substance, so that no comparisons could be made. This spot was also suspected to be IAM, but agreement with the reference substance in Solvent V was not very good (Rf. 0.62 as against Rf. 0.50). However, this possibility cannot be completely excluded.

O. Discussion of the auxin metabolism in *Exobasidium*

Now that the effects of the *Exobasidium* strains on various indole compounds have been investigated and discussed separately, I have tried to summarize the information obtained about their mutual relationships in the form of the scheme below. Auxin metabolism in *Exobasidium*—primarily in *E. myrt.* "Andr." 2—is thus, in general, as follows:



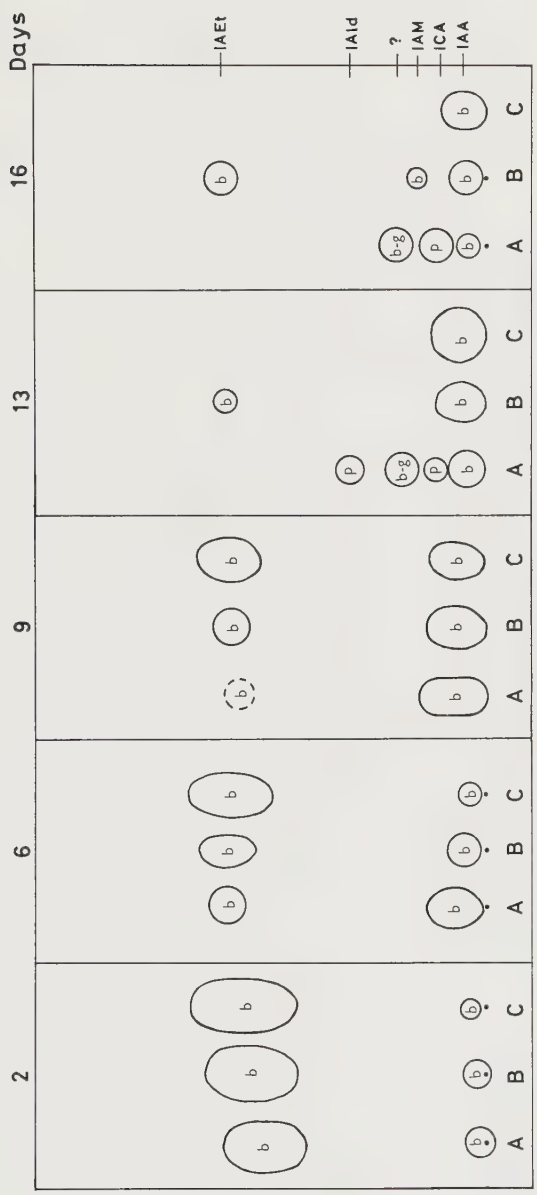


Fig. 39. The conversion of IAEt by *E. vacc.* "vit. id." 17 (A), *E. myrt.* "Andr." 2 (B) and *E. myrt.* "myrt." 2 (C). The cultures were extracted with ether at pH 2.9 after 2, 6, 9, 13, and 16 days. Chrom. in Solvent I. Ehrlich's reagent. b = blue, b-g = blue-green, p = pink.

Those steps in the sequence of reactions which I consider to be the normal ones are drawn with solid lines, whereas those of more doubtful significance are shown as dotted lines.

The synthesis of TTP from inorganic compounds has been discussed earlier, and it is probably quite certain that it occurs via An. A. In addition to the free TTP shown to be present in the plants, there is protein-bound TTP, which can probably be utilized by the fungi.

It is also certain that further synthesis after TTP is via IPyA, while the alternative pathway via tryptamine probably doesn't operate at all. This is in agreement with Perley and Stowe's (1966) opinion that this pathway is not open to fungi. However, it has clearly been shown that *E. myrt.* "Andr." 2 readily converts the exogenous tryptamine which is found in the host plant. However, in their ability to form IAA rapidly from tryptamine, *E. myrt.* "Andr." 1 and 2 are alone in the genus, while certain other systemic species can do so to a lesser extent after a time, and most of the localized spot-formers cannot do so at all. However, these latter form an unidentified substance (blue-green, Rf 0.18) which the host plants can probably convert to IAA.

The weaker traces of ILA found in the long term experiments with TTP medium and the total absence of this substance in short term experiments lead me to believe that synthesis of IAA from TTP does not go via ILA to a large extent, even if this pathway is open at all. This is the opposite of what happens in *Taphrina deformans* (Perley and Stowe, 1966) and *Diplodia natalensis* (Bailey and Gentile, 1962) in which significant amounts of IAA are formed rapidly. These authors, and Kaper and Veldstra (1958) have found indications that ILA is not formed directly from TTP, but from IPyA. Kaper and Veldstra, who worked with *Agrobacterium*, also found that ILA, together with tryptophol, were almost the only substances which were formed from TTP under anaerobic conditions. In these circumstances, ILA is obtained by reduction of IPyA, and tryptophol by reduction of IAAld. That such small amounts of ILA were found in *Exobasidium* might therefore be because the conditions during the experiment were definitely aerobic, since the flasks were on a shaker. It must also be remembered that the formation of IAA from ILA was so slow in my experiments that there is reason to doubt whether this reaction does occur naturally in *Exobasidium*.

Tryptophol has been clearly demonstrated, in experiments with IAAld, and also with IPyA, and its place in the scheme should be undisputable. However, it may be asked whether the amount of tryptophol formed by a dismutation reaction of IAAld can really be oxidized so rapidly as to leave no trace of the substance, for example in short term experiments with TTP, which otherwise gave clear spots, even for IPyA and IAAld.

It has gradually become apparent that *E. myrt.* "Andr." cultures are not the only ones which are able to form IAEt. However, there is no doubt that these, together with some of the other systemic species such as *E. Karstenii* cultures and *E. uvae ursi,* form considerably larger amounts of this auxin than do *E. vaccinii* cultures, for example. This must depend, in its turn, on the levels of IAA reached by the species, since it was apparent that large amounts of IAA also gave clear quantities of IAEt. However, in any one species, the level of IAA is determined by two factors, namely partly by the ability to convert the nitrogen-containing compounds rapidly and effectively to IAA, and partly by the effectiveness of the enzyme systems which immediately begin to break down the auxin. If this rough division into systemic species and species which form localized spots on the host can be used—in many the disease symptoms are difficult to interpret—then it is clear that the markedly systemic species rapidly form large amounts of IAA, which are only slowly broken down via IAld and ICA, whereas IAA synthesis in the species which form localized spots is slower and the oxidase activity is greater. Thus in addition to the effect which the rapidly-formed amounts of IAA themselves have on the morphogenesis of the host plant, an auxin reserve in the form of IAEt can be built up by the systemic species. When needed, this reserve can later rapidly be converted to IAA, so that availability of the auxin is always high, and can affect the host plant. I therefore consider that the rapidity and effectiveness of formation of auxins is a major contributory cause of the marked stimulation of growth of shoots and leaves of the host plant, brought about by the systemic *Exobasidium* species; whereas the lower production of auxin by the species forming localized spots may be one of the reasons why infections by such species are never so extensive.

My observation that IAA easily could be transformed into IAEt, and vice versa, agrees well with the investigations of the lethal effects of different IAA-esters on free cells of the Ehrlich ascites carcinoma. Schultz (1963) showed that IAA inhibited the speed of the breakdown of IAEt.

It is not only in *Exobasidium* that differences in auxin production have been observed. In a comparison of *Ustilago zeae* and *U. nigra,* Wolf (1952) has found that the former, which causes tumours in maize, forms large amounts of IAA, whereas *U. nigra,* which has no effect on its host, could not produce IAA on TTP medium.

Studies of the ability of parasites to increase growth of their host plants have in most cases shown that it depends on IAA production by the parasite, resulting in a higher concentration of IAA in the infected part of the plant. These observations have led to a number of conclusions (Brian 1957). Firstly, the parasite is for some reason more effective in converting the TTP of the host to IAA than the host's own enzymes; secondly, the IAA oxidases in

the host, which in uninfected tissue operate to hold the amount of IAA under a certain level, do not succeed in doing the same thing with IAA produced by the fungus. Assumptions that the increased amount of IAA in the tissues depends on reduced oxidase activity, caused for example by production of oxidase inhibitors by the parasite, seem not so probable, in any case for *Exobasidium,* because of the large amounts of IAA which the fungus can demonstrably form. Nor can one exclude the possibility that growth substances of other types can be, and probably are, formed by fungi (*see* ch. VIII). Curtis (1958) has deduced, from his investigation of the effect of filtrate from *Aspergillus niger* on bean plants, that IAA can be completely eliminated as the active substance causing deformation and growth of the plants.

To return to the scheme of auxin metabolism, it may also be said that IBA and other carboxylic acids with long side chains containing an even number of carbon atoms can serve as precursors of IAA, theoretically but probably not actually, but that this does not apply to acids with uneven numbers of carbon atoms in the side chains. IAM, however, probably takes part in the metabolism, because, for example, *Exobasidium* rapidly converts it to IAA, but whether it is a product of conversion of any other indole compounds is unknown. In any case, it is not a product of conversion of IAN, which to judge from the experiments, has no part in normal auxin metabolism. Thimann (1963) writes in his review that the very limited availability in the plant kingdom of enzymes which convert IAN to IAA seem to eliminate IAN as a precursor.

The question mark at the step in the reaction between ICA and indole is there because it is not fully confirmed that indole is formed in *E. myrt.* "Andr." 2. However, on TTP medium this species forms a substance which is probably indole (*see* Table 28). It is then possible that the circle is completed, so that the newly-formed indole can again combine with serine to form TTP, instead of undergoing further breakdown, for example to An. A, which could not itself be demonstrated as a breakdown product.

P. The auxin content of extracts from plant material infected by *Exobasidium*

Several authors have previously extracted plants with and without infections by fungi or other microorganisms to find out, by chromatography or bioassay, whether there is a higher auxin content in the infected plant tissues. Thus Hirata (1956) investigated several gall-forming fungi, including *Exobasidium camelliae,* and noticed that the greater part of the auxin in the

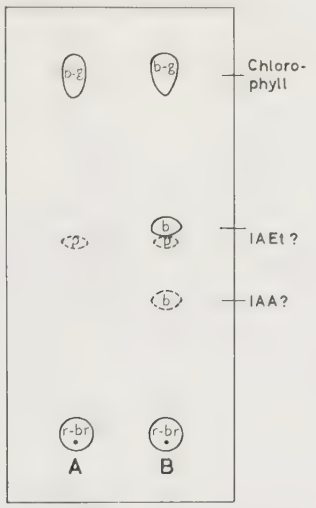


Fig. 40. Ether extracts (pH 2.9) of uninfected (A) and *Exobasidium*—infected (B) shoots of *Andromeda polifolia* were chrom. in Solvent S and sprayed with Ehrlich's reagent. b = blue, -g = blue-green, p = pink and r-br = red-brown.

gall tissue was produced by the parasitizing fungi themselves. In 1958 Daly and Inman made a quantitative comparison of the auxin content in ether extract from healthy and rust-infected *Carthamus* hypocotyls and found, in bioassays, that the zones on the chromatograms corresponding with the IAA position had considerably greater activity in promoting growth when the extracts were from infected tissues than when they were from healthy tissues, at all times during the period of infection. In investigations of tobacco and banana seedlings infected by *Pseudomonas solanaceum*, Sequeira and Kelman (1962) found a considerable increase in IAA and also in an unidentified growth inhibitor. Clarke *et al.* (1959) could detect no free IAA in extracts of up to 1 kg of healthy tomato stems, but found it, although in small amounts, together with ICA, in crown-gall tissue. These authors also consider that other non-acid growth substances could have been present and contributed to the extensive growth in the galls.

My own investigations have been made on *Exobasidium*-infected shoots of *Andromeda polifolia* (systemic infection) and leaves of *Vaccinium vitis-idaea* (localized spot infection). Despite long walks on boggy terrain it has proved difficult to find enough infected plant material for reliable auxin determinations. Also, other undesirable products are included in the extracts, for example chlorophyll, which prevent large amounts of extract being applied to the chromatogram and makes the interpretation of any spots more difficult.

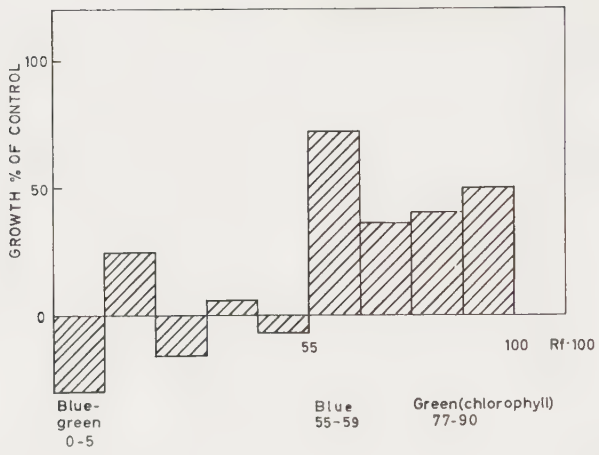


Fig. 41. The response of coleoptiles to parts of a chromatogram (Solvent S) of an ether extract of *Andromeda polifolia* infected by *Exobasidium*. Ehrlich's reagent.

Attempts to remove the substances with such a disturbing effect have so far not succeeded, in that the auxins have also disappeared from the extract. However, in spite of these difficulties some results have been achieved.

Fig. 40 shows a chromatogram of similar amounts of extracts from infected and uninfected *Andromeda polifolia*. Besides various chlorophyll spots, which appear in both extracts even before spraying with the reagent, there is a blue spot at Rf 0.50 in the extract from the infected shoots. The position and colour of the spot are the same as for IAEt. The healthy shoots do not contain this substance in demonstrable quantities.

In many cases the bioassays in which the chromatographed extract has been tested have given no results, because the amounts of the substances put on the chromatogram must be so low. However, Fig. 41 demonstrates some of the points which can most often be deduced from the results. The most important of these is that the blue spot at Rf 0.50 has significant auxin activity. The zone highest up on the chromatogram usually also has a stimulating effect on coleoptile growth, whereas the zone containing the starting point always seems to be inhibiting. The zone of the chromatogram which gave the maximum growth in this experiment has also been eluted and measured spectrophotometrically in UV. The result (Fig. 42) was a curve typical of indole derivatives, with a maximum at 282 nm, but unfortunately without any details.

In these investigations using ether extract no trace of IAA could be detected. However, when the extraction was with water, and the water was subsequently shaken with ether at pH 2.9, weak spots on the chromatogram

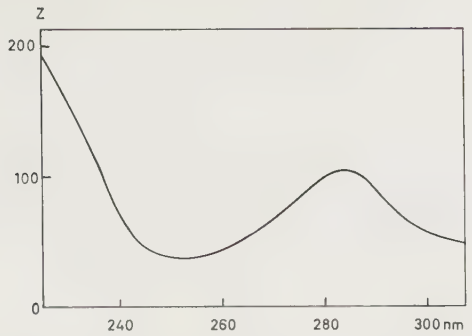


Fig. 42. UV absorption spectrum of the blue (Ehrlich's reagent) spot at Rf 0.46 on a chromatogram (Solvent S) of an ether extract of *Andromeda polifolia* infected by *Exobasidium*.

could be detected in the area where IAA is usually found. Table 41 also shows slight elongation of the coleoptiles when this zone was tested.

No growth-promoting substances have been found in extracts of leaves of *Vaccinium vitis-idaea* with gall swellings. Instead, there is usually a very intense blue-fluorescent spot on the chromatogram, indicating that there are large amounts of An. A. in these leaves. This interpretation is confirmed by the UV spectrum for the spot. This agrees with my observations that much larger amounts of An. A. ordinarily are found in cultures of gall-producing strains than in cultures of systemic ones. An accumulation of An. A. occurs, as has been shown earlier (p. 68), when the synthesis of IAA for some reason is blocked.

A number of attempts to bring about leaf enlargement and other symptoms of infection in the host plants by smearing them with lanolin paste containing biologically active zones from the chromatograms have also been made. The only detectable result obtained in this way has been that a number of the younger leaves have rolled up somewhat. Kiermayer (1958) made similar at-

Table 41. The effect on coleoptiles of parts of a chromatogram of aqueous extract of *Exobasidium*—infected shoots of *Andromeda polifolia*. Solvent S. Ehrlich's reagent.

Rf values	Colour with reagent	Growth % of control
0.00–0.10	blue-green	− 16
0.10–0.20	pink (weak)	− 6
0.20–0.30	0	− 3
0.30–0.40	blue (weak)	+ 18

tempts, to cause the stem of *Capsella bursa-pastoris* to bend in the way brought about by *Albugo candida,* by smearing on lanolin paste containing IAA. This was first successful when he used a 10% IAA solution. Lower concentrations such as 10^{-2} and 10^{-1} M were ineffective. The reason for this is certainly that only a small proportion of the auxin in the paste can get in through the epidermis, whereas the fungal hyphae grow in the tissues, so that the auxin they produce is taken up directly by the cells.

VI. INVESTIGATIONS OF THE ABILITY OF *EXOBASIDIUM* TO FORM PHENYLLACTIC ACID

Tamura and Chang (1965) reported that they had isolated L-β-phenyllactic acid from the culture broth of *Exobasidium symploci-japonicae*. This compound specially had a positive effect on the growth of roots, but a small elongation of the hypocotyls of lettuce seedlings was also observed. For that reason I have tried to find this substance in ethylacetate and ether extracts (pH 3.0) of *E. myrt.* "Andr." 1 and 2, *E. vacc.* "vit. id." 3 and *E. uvae ursi* 1. Those strains were cultivated in media containing NH_4NO_3, N-Z case or yeast extract. The extracts of the cultures were chromatographed (in Solvents S, IV and petrol. ether : chloroform : ethanol 50 : 30 : 20) and the chromatograms were analysed under UV-lamp, sprayed with reagents on phenols (among others the anisaldehydesulphuric acid reagent) and UV absorption spectra were measured. The results were compared with those of the reference substance.

In this investigation nothing has been found that indicates that phenyllactic acid should have been produced by the strains examined by me. The results must nevertheless be considered as preliminary. I have not had exactly the same medium as was used by Tamura and Chang (1965). Besides yeast extract theirs also contained 0.3% corn steep liquor.

VII. INVESTIGATIONS OF THE ABILITY OF *EXOBASIDIUM* TO FORM GIBBERELLINS

It has seemed natural also to investigate whether *Exobasidium* can form gibberellins, because these substances were first shown in filtrate from a fungus, *Gibberella fujikuroi,* which causes giganticism in rice. Later investigations of the ability of fungi to form gibberellins have, however, failed to give the same positive result. Thus Curtis (1957) has tested filtrate from 1500 fungal cultures on dwarf maize plants, without once finding growth stimulation, if *Gibberella* is excepted. In *Gibberella,* six of the thirteen hitherto (1965) isolated gibberellins have been found, but these have also been demonstrated in, for example, seeds of higher plants. According to Stuart and Cathey (1961), gibberellin A_4 can effectively stimulate the growth of leaf blades of *Phaseolus* and *Raphanus.*

The biological test materials which I used to detect the presence of gibberellins were wheat leaves, and dwarf maize mutants (d-5), which react even more specifically to gibberellins. The standard curve for different gibberellin concentrations showed that wheat leaves were already markedly affected (+24%) at 0.01 γ/ml and that maximum growth was at 1 γ/ml (Table 42). Table 43 shows that maize seedlings tested with solutions of concentrations 0.1–10.0 γ/ml response by increased growth which is in proportion to the added amounts of gibberellins. It may be seen that it is primarily the length of the second leaf sheath which increases most. An experiment was designed to find out if gibberellins could really be extracted by the fairly complicated procedure described on p. (12), and how much was needed to cause a positive response in maize seedlings. Extractions were made from eight cultures of *Exobasidium* and two flasks with 10 and 1000 γ gibberellic acid. The aqueous solution which remained after shaking with active charcoal

Table 42. The effect of gibberellic acid (GA) on the growth of wheat leaf sections.

Conc. GA γ/ml	0.01	0.1	1.0	10.0	100.0
Growth % of control	+24	+27	+33	+15	+9

Table 43. The quantitative response of dwarf corn (D_5) 8 days after application of GA to the first unfolding leaves.

	Conc. GA γ/ml			
Length (mm) of	0	0.1	1.0	10.0
1st leaf-sheath	15	22	28	36
1st + 2nd leaf-sheath	18	67	93	122

was also tested, to see if all the gibberellic acid had been removed from the solution. Table 44 shows that it had been. There seemed to be no trace left in the original solutions, whereas even the extract from the flask with 10 γ could stimulate growth of the mutants. However, the fungal extracts had no effect on them.

Chromatography of gibberellins in isopropylether: acetic acid 95:5 has also proved successful, and the gibberellic acid moves to ca. Rf 0.11. Biological tests using the zones from the chromatograms therefore give positive responses for the 1st and 2nd zones of the ten. However, no such effect has been clearly apparent, in these zones or in any other, even though extracts of up to 5 litres of culture filtrate have been used in the experiments. Since most of the *Exobasidium* species used in the investigation have been tested, I consider that it is highly improbable that the observed growth stimulation of the host plants is caused by gibberellins.

Table 44. The quantitative response of dwarf corn (D_5) 6 days after application of fungal extracts and extracts of GA.

	Length (mm) of	
Extract from	1st leaf-sheath	1st + 2nd leaf-sheath
E. Karst. 3	10	13
E. cass. 1	10	14
E. vacc. "vit. id." 6	7	10
E. vacc. "vit. id." 17	12	15
E. myrt. "vit. id." 3	15	17
E. myrt. "vit. id." 5	14	17
E. myrt. "myrt." 2	12	14
GA 10 γ	27	34
GA 1000 γ	40	70
Water-rest, GA 10 γ	15	17
Water-rest, GA 1000 γ	10	13

VIII. INVESTIGATIONS OF THE ABILITY OF *EXOBASIDIUM* TO FORM CYTOKININS

A. Results of callus tests

The third large group of plant hormones comprises the kinins, of which kinetin is the best-known. Kinetin was first isolated from autoclaved DNA; it stimulates cell division. Kuraishi and Okumura (1956) were among those who showed that kinetin affects both the fresh weight and area of leaves, effects which were also shown by my own experiments with *Raphanus* leaves. That kinetin-like substances with a hormone effect also occur naturally in plants has been shown in recent years by Letham *et al.* (1964) and Whitham and Miller (1965), for example. These authors, independently, have worked out the chemical formula of an aminopurine (zeatin), which is present in especially large quantities in unripe maize fruits, but also in sunflower seeds, soja beans and plums. However, I know of no report that fungi are able to form kinetin-like substances. A possible indication that *Exobasidium* might do so has arisen through Wolf and Wolf's (1952) histological studies of leaves infected by *Exobasidium camelliae,* which showed that besides hypertrophy (enlargement through increased cell size), hyperplasy (enlargement through increased cell division) is involved.

In my experiments I have mainly used *E. uvae ursi,* which induces copious shoot formation by its host plants and because of this can be supposed to induce cell division. *E. myrt.* "Andr." 2 has also been tested. The experimental material was tobacco callus, specially isolated and cultured for the purpose. Kinetin was omitted from Medium 5 in which the callus tissue grew during the experiments, while auxin in the form of IAA was included.

The following results are shown by Table 45. On the first occasion when measurements were taken about one month after the beginning of the experiment, the amount of growth was assessed on a scale with five divisions. As expected, growth on that occasion was greatest in the flasks containing kinetin, while it was fairly moderate in the flasks with additions of ether extract of *E. myrt.* "Andr." 2 and somewhat greater with extract of *E. uvae ursi* 2, similar to that in only adenine medium without fungi. Almost 2 months later the clumps of callus had grown quite a lot in most of the flasks. Also on this occasion the most growth in the flasks containing fungi had been

Table 45. An assay with tobacco callus, to which ether extracts (pH 7.0) of *E. myrt.* "Andr." 2 and *E. uvae ursi* 2 on adenine medium (17 days) were added. Three replicates.

Extract of	Estimated growth after 1 month	Estimated growth after 3 months
Adenine medium without fungus	+	+ +
E. myrt. "Andr." 2	+	+ + +[a]
E. uvae ursi 2	+ +	+ + + +[b]
Complete callus medium with kinetin(= Medium 5)	+ + + + +	+ + + + +

[a] 1 shoot with leaf after 4 months.
[b] 2 shoots with leaves after 3 months.

in those with extract from *E. uvae ursi* 2, but growth was also good in those with extract from *E. myrt.* "Andr." 2. What was perhaps more noteworthy was that in two of the *E. uvae ursi* flasks short shoots with green leaves had been formed. These are shown in Plate 4. Still later, a similar leaf rosette had been formed in *E. myrt.* "Andr." 2. Shoot formation in the same isolation of tobacco callus, which I used, has been brought about with kinetin by Fil. lic. Gunnar Fridborg at this Institute (unpublished data). 0.04 mg/l kinetin gave some shoots, but the optimum concentration was about 0.4 mg/l.

Extract from *E. uvae ursi* 2 was also used in the next experiment. The filtrate was first extracted in the usual way at pH 7.0, then it was boiled and extracted again. This was done to see if any new active substances were formed as a result. An aqueous solution of kinetin was also extracted at pH 7.0. Then the three extracts were chromatographed in Solvent S. The following results were obtained with the zones scraped from the chromatogram (Table 46). It is clear that kinetin can be extracted by the method used, and that the kinetin spot lies mainly between Rf 0.00 and 0.20. The callus tissue also responds clearly to this zone and has its highest growth rate. The same tendency is apparent for the flasks with chromatographed fungal extract, i.e. the maximum fresh weights lie within zones 1 and 2, and the fresh weights decrease successively towards higher Rf values. Here also there was a shoot with leaves formed, exactly in the first zone for extract from the boiled filtrate of *E. uvae ursi* 2.

Naturally, more detailed studies (*see* below) were necessary before anything definite could be said about production of kinin by *Exobasidium*. However, there was some evidence of a substance which stimulates cell division. The shoot formation which took place certainly indicated this.

Table 46. An assay with tobacco callus, to which scraped-off parts of a chromatogram (in S) was added. Ether extracts (pH 7.0) of unboiled and boiled culture filtrate of *E. uvae ursi* 2 on adenine medium (17 days). Experiment duration 2 months.

Extract of	Rf values	Mean fresh weight, mg
E. uvae ursi 2		
Unboiled filtrate	0.00–0.20	713± 35
	0.20–0.40	875± 58
	0.40–0.60	275± 40
	0.60–0.80	252± 32
	0.80–1.00	190± 25
Boiled filtrate	0.00–0.20	488± 41[a]
	0.20–0.40	343± 45
	0.40–0.60	157± 20
Kinetin	0.00–0.20	1621±155
	0.20–0.40	449± 51
	0.40–0.60	289± 39
Adenine medium without fungus	0.00–0.20	264± 42

[a] Shoot with leaf.

Since it has earlier been shown that *Exobasidium* can form a large number of auxins, if kinetin production also takes place then the conditions for bringing about a marked stimulation of growth of the host plants are fulfilled. However, it might well be that the ability to form substances which stimulate cell division is restricted to *E. uvae ursi,* but the results obtained in the above experiment with *E. myrt.* "Andr." 2 indicate otherwise (the shoot formation).

B. Results of oat leaf tests, wheat root tests and spectrophotometric measurements

To see whether it was possible to obtain a clearer picture of *Exobasidium's* ability to produce cytokinins, the following experiments were later carried out, using the oat leaf and wheat root tests. The tested *Exobasidium* cultures (*E. vacc.* "vit. id." 3, *E. myrt.* "Andr." 2 and *E. uvae ursi* 2) were grown on asparagine as nitrogen source. Chloroform-extracts from these cultures were tested in the oat leaf test and gave positive results, i.e. chlorophyll reten-

Table 47. Optical density (OD) at 660 nm of acetone extracts of oat leaves, which had been treated with chloroform extracts (in 0.2% Tween 80) of fungal cultures (14 days) or of kinetin.

Test material	Control = 0.2 % Tween 80	*E. myrt.* "Andr." 2	*E. uvae ursi* 2	*E. vacc.* "vit. id." 3	Extract of kinetin	
					Chlorof. fraction	Aqueous fraction
Z value	81	123	112	86	128	81

tion, for *E. myrt.* "Andr." 2 and *E. uvae ursi* 2 but a negative result for *E. vacc.* "vit. id." 3. These results were reproducible in subsequent experiments. Ten ml 10^{-7} M kinetin solution was extracted with chloroform and tested. The chloroform fraction gave a positive result whereas the residual, aqueous fraction gave a negative result, showing that the method of extraction was suitable. The visual observations were in general agreement with values obtained by measuring the optical density of acetone extracts of the oat leaves (Table 47).

It had previously been shown that *E. myrt.* "Andr." 2 and *E. uvae ursi* 2 could synthesize auxins when grown on asparagine as a nitrogen source, and it was therefore necessary to chromatograph the extracts to exclude the possibility of any such auxins affecting the result of the oat leaf test. In most cases my investigations of the sensitivity of this test to various known substances have agreed fairly well with Thimann and Sachs' (1966) results. For example, the lowest concentration in which kinetin could be detected was 0.5×10^{-7} M; for adenine it was 10^{-2} M; for asparagine, clearly detectable effects were obtained only at concentrations of 0.3 M or more. In contrast, I found that IAA and IAEt were considerably more effective than Thimann and Sachs had found (10^{-5} M was detectable, instead of 1.7×10^{-4} M). Tests of the three lowest zones (Rf 0.00–0.10; 0.10–0.20; and 0.20–0.30) in Solvent S of the chromatogram with *E. myrt.* "Andr." 2, *E. uvae ursi* 2 and kinetin, demonstrated that the positive result given by *E. myrt.* "Andr." 2 extract could be attributed to the IAA, in zone 3. Small, distinct, green spots remained under the drops from zone 3, but not from zones 1 or 2. For the same reason, small green spots remained under drops from zone 3 of the extract from *E. uvae ursi* 2, but there was also some effect of drops from zones 1 and 2, as there was for the chromatographed kinetin. Leaves used in this experiment were damaged to some extent by the small quantities of the solvent remaining in the scraped-off silica gel.

This experiment led to the conclusion that *E. uvae ursi* 2 was the only

Table 48. The result of an oat leaf test of a chloroform extract (pH 7.0) of *E. uvae ursi* 2 grown on NH_4NO_3 + adenine medium (14 days). Solvent S.

Rf values	–0.05– 0.05	0.05– 0.15	0.15– 0.25	0.25– 0.35	0.35– 0.45	0.45– 0.55	0.55– 0.65	0.65– 0.75	0.75– 0.85	0.85– 0.95	0.95– 1.00
Chlorophyll retention	+ +	+ +	–	+	–	+	–	–	–	–	–

one of the tested *Exobasidium* species which might produce cytokinin on an asparagine medium. This fungus was therefore grown on three litres of the same medium (as opposed to 1 l, previously). The fungal material was centrifuged off, and hydrolyzed ($^1/_2$ hour in boiling 1 N HCl) and a chloroform extract was chromatographed, together with a chloroform extract of the medium.

Both extracts gave only three spots which appeared dark when the silica gel, containing UV indicator, on the chromatogram was illuminated with UV light. In Solvent IV (chloroform : CCl_4 : methanol 50 : 25 : 25) their Rf values were 0.08, 0.60 and 0.80. Solvent IV appeared very suitable for separations of this type. The spot at Rf 0.08 was very small and had no effect in the oat leaf test. It was almost certainly IAA in a very low concentration, and this would be expected, since the extraction was at pH 7.0. The spot at Rf 0.80 was largest. It was particularly dominant in the extract of the hydrolysed fungus and was asparagine, at least in the main. The spot at Rf 0.60 was medium-sized and was the most interesting because it had an Rf value similar to that of kinetin and it had a clear effect on the oat leaf. However, chromatography in five different solvents and determination of its UV spectrum showed clearly that this substance was tryptophol. Since no other part of the chromatogram of the extract caused chlorophyll retention in the oat leaf, I conclude that it is unlikely that *E. uvae ursi* 2, or any of the other *Exobasidium* species, produces cytokinin on asparagine medium.

Like the previous experiments with tobacco callus, the new bioassays have been used to test extracts from fungi grown on culture media containing adenine. The fungi grow on media with adenine as the only nitrogen source, but not very well, and the most usual combination was NH_4NO_3 + adenine. Uninoculated media with NH_4NO_3 + adenine, or similar volumes with NH_4NO_3 or adenine alone, were used as controls.

Table 48 shows that there were two zones causing distinct chlorophyll retention and two causing less distinct retention. The weaker positive results caused by zones Rf 0.25–0.35 and 0.45–0.55 are almost certainly attributable to traces of IAA and IAEt, respectively. The positive effects of zones – 0.05

Table 49. Oat leaf test of a chloroform extract (pH 7.0) of *E. uvae ursi* 2 grown on NH_4NO_3 + adenine medium (14 days). Kinetin was added to the starting point of one chromatogram. Solvent IV. Visual observations of chlorophyll retention, and OD at 660 nm of the acetone extracts of the leaves.

		E. uvae ursi 2				
Test material and Rf values	Control = 0.2 % Tween 80	0.00–0.10	0.40–0.50	0.60–0.70	+ kinetin 0.00–0.10	+ kinetin 0.40–0.50
Z value	96	100	195	190	108	670
Chlorophyll retention	–	–	+	+	–	+ + +

to + 0.05 and 0.05–0.15 might be due to a kinetin-like substance. In this solvent (S), chromatographed kinetin has a Rf value of 0.05. Some adenine from the medium is unfortunately present in the chloroform extract. It remains at the starting point on the chromatogram and might possibly be the explanation of these results. However, the leaves react to adenine only when it is present in concentrations of 10^{-2} M or more.

The two lowest zones on the chromatogram were also tested using wheat roots. Corresponding regions of the chromatogram of the extract from the same volume of uninoculated medium were used as a control. Previous preliminary experiments had shown that the roots reacted clearly to kinetin in concentrations as low as 10^{-8} M; and that some effects were discernible even at 10^{-9} M. Reactions more or less identical to those with kinetin were obtained with IAA and IAEt, but at higher concentrations. Fairly distinct thickenings and curvatures developed in the roots of most of the seedlings used to test zone − 0.05 to + 0.05 of the chromatogram of the extract from *E. uvae ursi* 2. If this effect was entirely attributable to adenine it would also have been caused by the same zone from the extract of the uninoculated medium. This was not so, nor was there any clear effect of any of the other zones. The magnitude of the reaction caused by the active zone was about the same as that caused by 10^{-8} M kinetin.

In the following experiments using oat leaves, Solvent IV was used instead of Solvent S, which is not always suitable, to separate adenine from kinetin. In Solvent IV, adenine has Rf 0.19 and kinetin 0.47. There are sometimes traces of kinetin left just after the starting point on the chromatogram and this zone was therefore included in the experiments, the results of which are shown in Table 49.

Positive results were obtained for zone 0.40–0.50 of the fungal extract,

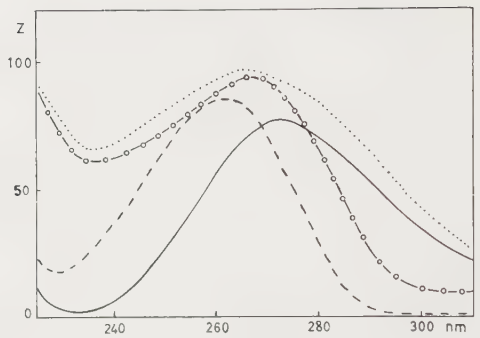


Fig. 43. UV absorption spectra of region 0.20–0.30 of a chromatogram (Solvent IV) of an ether extract of *Arctostaphylos uva-ursi* (————) and region 0.90–1.00 of a chromatogram (Solvent isopropanol: NH_3: H_2O 85:5:15) of a neutral ether extract of *E. myrt.* "Andr." 2 on NH_4NO_3 medium for 15 days (·····). Chrom. (Solvent IV) adenine (— — —) and kinetin (○—○—○—○) are shown for comparison.

both with and without added kinetin. The green spots retained under the drops from the chromatogram of fungal extract alone were smaller than those under drops from the sample with added kinetin, but the extinction value was definitely above that of the control. No substance of the auxin type could be detected in this region, although at Rf 0.70 a blue spot was obtained after treatment with Ehrlich's reagent. This spot was certainly IAEt and is the cause of the positive reaction in the oat leaves treated with chromatogram zone 0.60–0.70. Tryptophol has not been shown to be present in this extract and its Rf value is too high for it to be responsible for the effect of zone 0.40–0.50.

My results invite comparison with those obtained by Sommer (1961) with *Taphrina deformans*. This fungus resembles certain *Exobasidium* species in that it causes neoplastic growth in peach leaves. Sommer (1960) had shown that IAA is produced by the fungus and he later demonstrated the occurrence of one or several cytokinins in very old (4–6 months) cultures of *Taphrina* on potato dextrose medium. However, Sommer used paper chromatograms in his tests and Salkowski reagent, which is not very sensitive, to show the presence of auxins.

Investigations of *Exobasidium*-infected material of *Arctostaphylos uva-ursi* have also been made. About 30 infected, red-coloured shoots, and a similar number of controls, were ground up and extracted with ether at pH 7.0. The extracts were chromatographed in Solvent IV and UV measurements were made of the various zones on the chromatograms. No kinetin-like spectrum was obtained in zone 0.40–0.50, although this might have been expected. However, for zone 0.20–0.30 of the extract from the infected shoots the UV

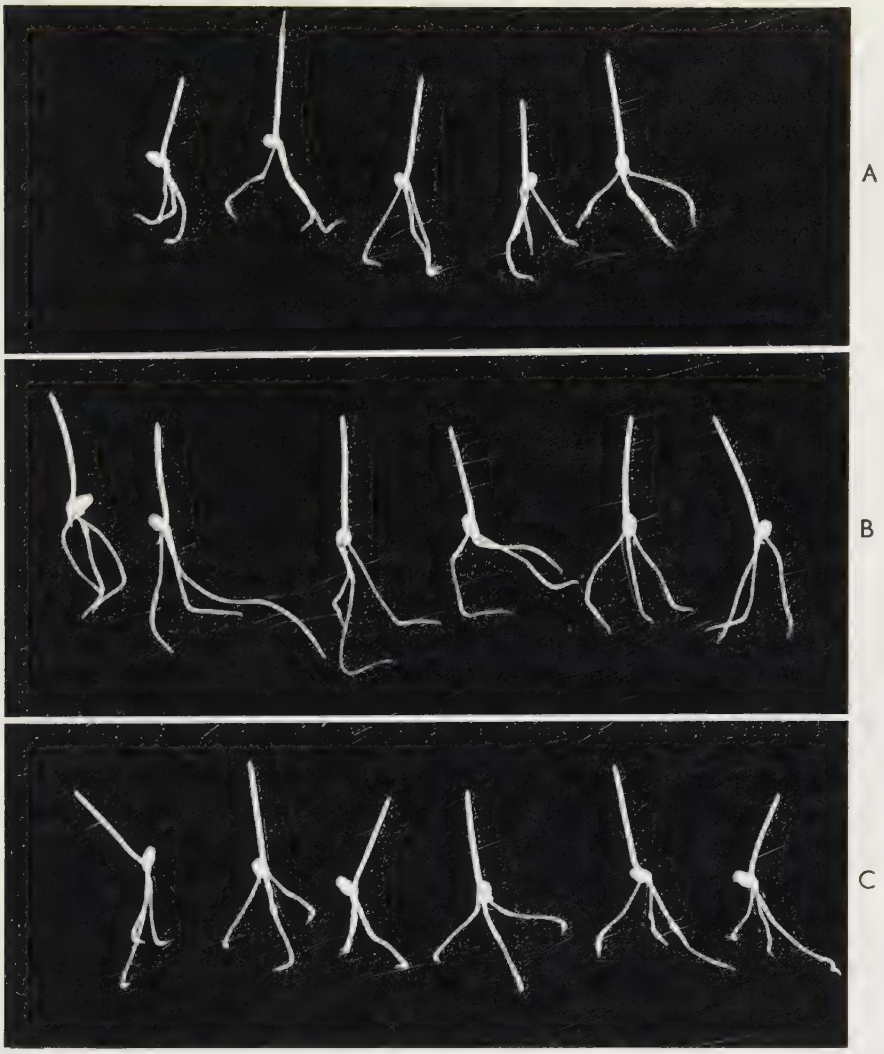


Photo. 2. The response of wheat roots to region 0.20–0.30 of a chromatogram (Solvent IV) of ether extract of *Arctostaphylos uva-ursi* (A), compared with untreated control (B) and the effect of kinetin (10^{-7} M) treatment (C). Ca. natural size.

curve drawn in Fig. 43 was obtained. The curve has a minimum at 234 nm and a maximum at 272 nm, and in these respects is seen to be somewhat different from a kinetin curve. However, the UV spectrum has a typical purine-like appearance and in several respects is very like some of the curves measured by Shaw and Srivastava (1964) for substances which they had isolated

from coconut endosperm, and which also caused chlorophyll retention in leaves.

The UV spectrum of adenine has been included in Fig. 43 together with the curve for an unknown substance in zone Rf 0.90–1.00 of a chromatogram (in isopropanol : NM_3 : water 85 : 5 : 15) of a neutral extract of *E. myrt.* "Andr." 2 grown on NH_4NO_3 medium. The curve for this unknown substance had a minimum at 235 nm and a maximum at 265 nm and is therefore in close agreement with the curve for kinetin but is very different from that characteristic of indole compounds. This perhaps explains a previous result (*see* p. 99), when in tests with tobacco callus a shoot was developed on callus in a flask containing extract from *E. myrt.* "Andr." 2.

The methanol containing the substance from *Arctostaphylos uva-ursi,* indicated to be a cytokinin by its UV spectrum, was evaporated off after the UV measurements and tests with wheat roots were carried out. The results are shown in Photo 2, which shows the clear thickenings and curvatures on the roots caused by the substance as compared with the control. Testing zone 0.20–0.30 from a new chromatogram gave the same result. It seems, therefore, that plants infected with *E. uvae ursi* contain cytokinins in the same way as fungal cultures on media containing adenine. The apparent requirement for adenine or closely related substances for the synthesis of cytokinins, at least in detectable amounts, probably would not affect the fungus in vivo, since the host plant could provide such substances. It is of course also possible that the host itself synthesizes the cytokinins, because of the influence of the fungus. Another possibility is that the active substance is not kinetin, but some closely related compound. Sommer (1961) also suggested this as a result of his work with *Taphrina.*

SUMMARY

Using thin layer chromatography, investigations of the production of growth-stimulating substances by *Exobasidium* have been made on culture extracts of various species of the genus and on extracts of infected plant material. The biological effect of substances found in this way, and of known substances, has been measured in leaf and coleoptile tests. More detailed information about the identity of unknown substances has been obtained from spectrophotometric measurements.

All the *Exobasidium* species studied could form indole compounds on media containing only an inorganic nitrogen source. However, production was considerably higher when organic nitrogen sources were present.

Auxin metabolism on tryptophan medium was studied in special detail and the various steps in the reaction sequence have been followed up by incubation experiments with the various metabolites. In the same way, information as to which synthetic pathways are normal in *Exobasidium* has been obtained.

Exobasidium forms two main indole compounds with high auxin activity, viz. indoleacetic acid (IAA) and IAA ethyl ester (IAEt).

In extracts of *Exobasidium*-infected shoots, one main substance has been found, and this is very probably IAEt.

In aerobic conditions the synthesis of IAA is mainly via indolepyruvic acid and indoleacetaldehyde, two labile compounds, which have, however, been clearly shown. The synthetic pathway via tryptamine seems not to operate, but this does not prevent some *Exobasidium* species from being able to convert tryptamine to IAA easily. Formation of IAA via indolelactic acid has been shown to proceed very slowly.

Quantitative calculations have yielded the result that the maximum amount of IAA can be up to 11.7% of the added amount of DL-tryptophan in *E. myrt.* "Andr." 2, which is one of foremost auxin producers.

Distinct differences have been found in the ability of various *Exobasidium* species to form auxins. One group, mainly comprising species which infect the host plants systemically, forms large amounts of IAA rapidly, and, because the IAA oxidase effect is small, these are only slowly broken down to indolealdehyde and indolecarboxylic acid. Instead, IAA is converted to IAEt, which can be regarded as an easily utilized store of auxin. The second

group, which mainly comprises species which cause only small galls on the leaves, synthesizes IAA more slowly and the amounts are never so large, because breakdown is more effective here.

Investigations of the ability of *Exobasidium* to form gibberellins and phenyllactic acid have shown that these are probably not formed.

On the other hand, some kinetin-like substance may be produced, in any case by the two strains which cause witches' brooms on their hosts, as indicated by a certain amount of stimulation of callus growth with shoot formation, chlorophyll retention in leaves, root tests and UV-spectra.

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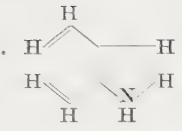
FORMULAE AND ABBREVIATIONS OF SUBSTANCES NAMED

R = indole ring.

Indole.



Skatole.
$R—CH_3$

3-Indoleacetic acid (IAA).
$R—CH_2—COOH$

3-Indolecarboxylic acid (ICA).
$R—COOH$

3-Indolepropionic acid (IPrA).
$R—CH_2—CH_2—COOH$

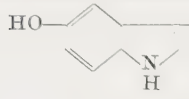
3-Indolebutyric acid (IBA).
$R—CH_2—CH_2—CH_2—COOH$

3-Indolelactic acid (ILA).
$R—CH_2—CHOH—COOH$

3-Indolepyruvic acid (IPyA).
$R—CH_2—CO—COOH$

3-Indolealdehyde (IAld).
$R—CHO$

5-Hydroxy-3-indoleacetic acid (OH-IAA).



3-Indoleacetaldehyde (IAAld).
$R—CH_2—CHO$

3-Indoleacetonitrile (IAN).
$R—CH_2—CN$

Ethyl 3-indoleacetate = IAA ethyl ester (IAEt).
$R—CH_2—COO—C_2H_5$

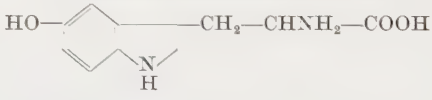
Tryptophol.
$R—CH_2—CH_2OH$

Tryptamine.
$R—CH_2—CH_2NH_2$

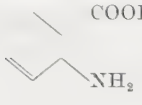
3-Indoleacetamide (IAM).
$R—CH_2—CONH_2$

Tryptophan (TTP).
$R—CH_2—CHNH_2—COOH$

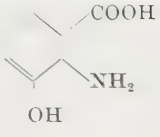
5-Hydroxy-tryptophan (OH-TTP).



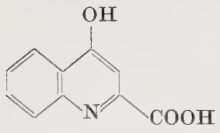
Anthranilic acid (An. A).



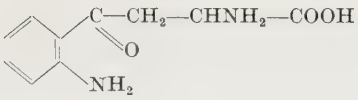
3-Hydroxy-anthranilic acid (OH-An. A).



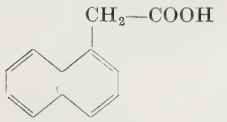
Kynurenic acid.



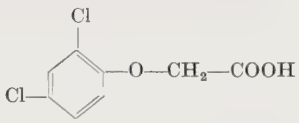
Kynurenine.



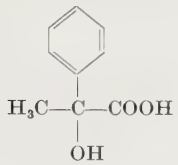
Naphthyl-1-acetic acid (NAA).



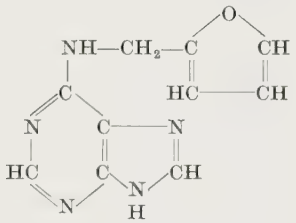
2,4-Dichlorophenoxyacetic acid (2,4-D).



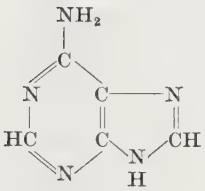
2-Phenyllactic acid.



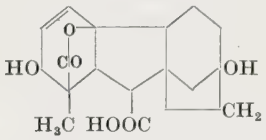
Kinetin. (6-furfuryl aminopurine).



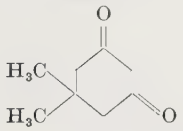
Adenine.



Gibberellic acid (gibberellin A_3) (GA).



Dimedone.



Colour reactions with some of the reagents, and Rf values in different solvents

	Reagent				Solvent		
					S	Sp	A
				H_2O: conc. H_2SO_4	Chlor.: acet. acid	Chlor.: methanol: acet. acid	Meth. acet.: isoprop.: NH_3
Substance	Ehrlich's	DNPH	van Eck's	30 : 70	95 : 5	75 : 20 : 5	45 : 35 : 20
IAA	blue-lilac	red-brown (slow)			0.32	0.78	0.32
ICA	pink (slow)				0.29	0.77	0.30
IPrA	blue				0.34		0.38
IBA	blue				0.38		0.40
ILA	blue				0.02	0.24	0.21
IPyA	brown-lilac	yel.-brown			0.00–0.12		decomp.
OH-IAA	blue				0.05	0.45	0.23
An. A	yellow (slow)				0.40		0.46
OH-An. A	orange (slow)				0.10	0.75	0.31
Skatole	blue				0.78		0.94
Indole	red-violet				0.74		0.93
IAld	pink (slow)		yellow		0.21	0.71	0.80
IAAld	brown-lilac	brown	red-violet		0.46		
IAN	red-violet				0.48		0.87
IAEt	blue				0.52	0.95	0.90
Tryptophol	blue				0.28		0.93
Tryptamine	blue-green				0.00	0.10	0.82
IAM	blue-lilac				0.11	0.63	0.85
TTP	blue-green				0.00	0.05	0.21
OH-TTP	blue				0.00	0.08	
Kynurenic acid	pale yellow						0.48
Kynurenine	yellow-br.					0.11	0.26
Adenine					0.00		
Kinetin	pale pink				0.05	0.65	0.80
Gibberellic acid				dark br.			
Chromatography time, min.					19	25	35

Isoprop.: NH_3 : H_2O 85 : 5 : 10	Petr. eth.: chlor.: ethan. 96% 50 : 30 : 20	I Chlor.: CCl_4: methanol 50 : 40 : 10	III Eth. acet.: isoprop.: H_2O 65 : 24 : 11	IV Chlor.: CCl_4: methanol 50 : 25 : 25	V Chlor.: ethan. 96% 65 : 35	Ethan. 50%. HAc-saturated atmosphere	Isopropyl-ether: acet. acid 95 : 5
0.38	0.07	0.02	0.27	0.08	0.11	0.72	
0.33	0.15	0.07	0.58		0.32		
0.19		0.01	0.06		0.00		
decomp.		0.00			0.02	0.68	
0.16			0.15				
		0.05			0.20		
		0.59			0.82		
		0.57			0.80		
0.71	0.26	0.30			0.74		
	0.60	0.35					
0.80	0.65	0.49			0.83		
		0.62	0.78	0.70	0.85		
0.73	0.52	0.28			0.68		
		0.00			0.05		
0.64	0.32	0.13			0.49		
0.12		0.00			0.02		
			0.01		0.07		
		0.06		0.19	0.15		
		0.27		0.47	0.52		
							0.11
80	21	20	45	25	27	45	18

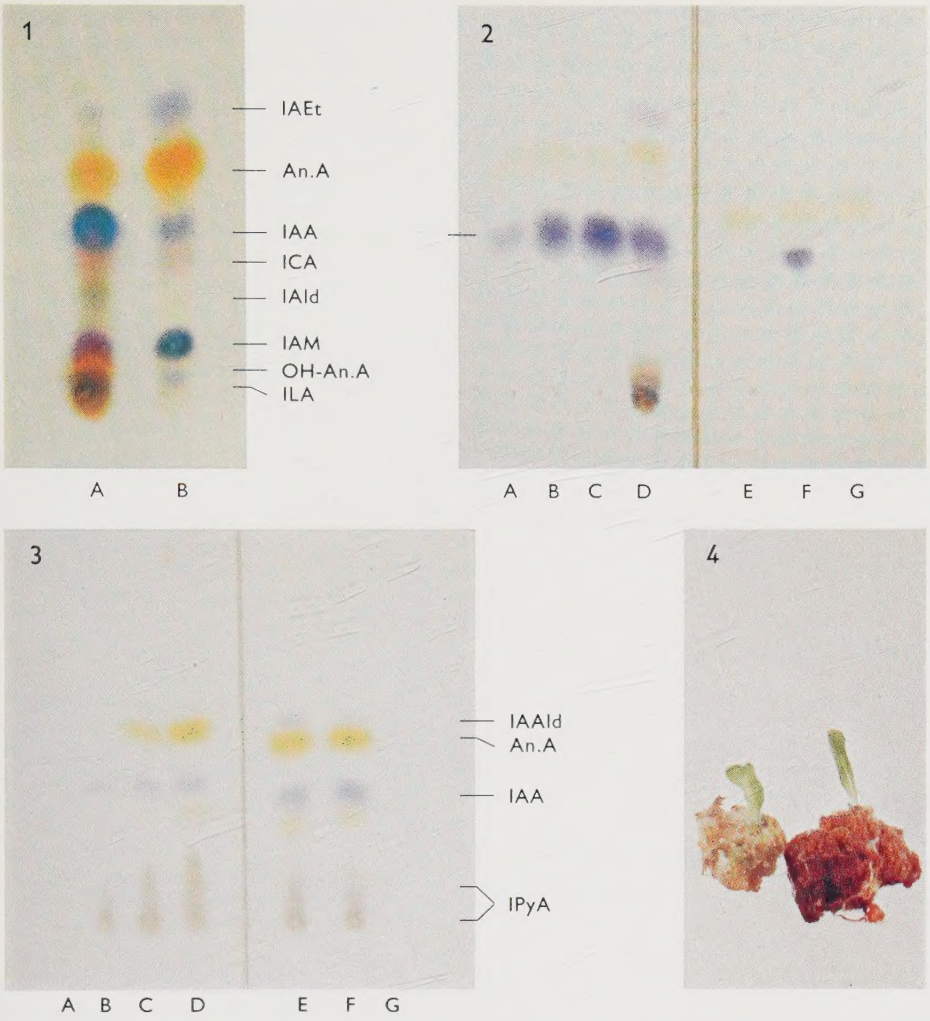


Plate 1. demonstrates the indole compounds in the combined ether extracts (acid and neutral fractions) of a 6 days old culture of *E. myrt.* "Andr." 2 on TTP medium (A). At B IAEt, An.A, IAA, ICA, IAM and ILA have been run as standards. Solvent S. Ehrlich's reagent.

Plate 2. 0.05 ml of ether extracts (pH 2.9) of known IAA concentrations were chromatographed together with extracts from 9 days old cultures of *E. myrt.* "Andr." 2 grown on TTP medium (D) and NH_4NO_3 medium (G). A = 0.1, B = 0.5, C = 1.0, E = 0.01 and F = 0.1 mg IAA per 50 ml culture medium. Solvent S. Ehrlich's reagent.

Plate 3. A demonstration of the auxin metabolism in a short term experiment with *E. myrt.* "Andr." 2 on TTP medium. Ether extracted at pH 2.9. Solvent S. Ehrlich's reagent. A and G = Controls without fungus, 1 and 12 hours old, respectively, B = 1, C = 3, D = 6, E = 9 and F = 12 hours old cultures.

Plate 4. Shoots with leaves on tobacco callus, formed in flasks with extracts (pH 7.0) from *E. uvae ursi* 2 on adenine medium.

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